Physiological and genetic studies of an alternative semi-dwarfing gene *Rht18* in wheat

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Transfer and assessment of the *Rht18* dwarfing gene from durum into bread wheat

G.J. Rebetzke, T. Tang, R.A. Richards, W.D. Bovill, A.G. Condon, A.R. Rattey, M. Ellis, and W. Spielmeyer

Prepared for submission to Field Crops Research

Fine mapping and development of SNP markers for *Rht18* in durum wheat

T. Tang, J. Hyles, W. Spielmeyer *et al.*

Prepared for submission to theoretical and applied genetics

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Abstract

Conventional semi-dwarfing genes *Rht-B1b* and *Rht-D1b* have successfully improved grain yield of wheat. This study investigates the physiology and genetics of a new semi-dwarfing gene, *Rht18*. Isogenic lines including Tall, semi-dwarf (*Rht-D1b/B1b* or *Rht18*) and Double dwarf (*Rht18+Rht-D1b/B1b*) were developed. There was no difference in developmental stage between semi-dwarfs from the time of terminal spikelet to maturity. However, *Rht18* slowed the growth of the spike and distal internodes to reduce plant height by 20-35% compared with Tall lines. Dry matter was relocated from the stem to the spike post-anthesis, and semi-dwarfs relocated less on a whole stem basis, but more per unit length compared with Tall. *Rht18* and *Rht-D1b/B1b* had similar spike weight, grain number, grain size and harvest index.

Rht18 maintained long coleoptile length in both bread and durum wheat. Coleoptile length was positively correlated with emergence percentage when sown deep under controlled conditions and in the field, indicating the potential for *Rht18* to replace *Rht-D1b* in future cultivars to improve crop establishment of wheat. In terms of seedling leaf area and biomass, *Rht18* has no advantage to *Rht-D1b*/*B1b* and both performed poorer than Tall. There was no evidence that *Rht18* has any effect on seed dormancy.

Genetic studies in durum wheat mapped *Rht18* to chromosome 6AS and a co-segregating SNP marker (*csRht18-SNP*). The *Rht18* associated allele of *csRht18-SNP* was absent in a wide range of international bread wheat germplasm. Allelism tests established that *Rht18* is most likely allelic to *Rht14*, but not to *Rht16*. Further mapping studies of *Rht14* confirmed this result, and located *Rht16* on chromosome 5B. The SNP marker tightly linked to *Rht18* will assist wheat breeders who aim to replace *Rht-B1b* and *Rht-D1b* with *Rht18*. The study also suggests that future cultivars with *Rht18* are likely to have longer coleoptiles and better emergence in water-limited and high soil temperature regions.

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Chapter 1 General introduction

1.1 Introduction

Wheat is one of the major food crops in the world and the demand for it will increase as the population grows and wealth increases in developing countries. Gains in grain yield in the past have been achieved largely through a higher harvest index (HI) (Jain and Kulshrestha 1976). HI was defined by Donald (1962) as the ratio of grain to total above ground biomass. Tall wheats under favourable conditions can reach heights of 150-170 cm, but they will typically lodge, particularly under optimum nitrogen regimes. Lodging reduces grain yield and causes many detrimental effects such as slower harvest, greater grain drying costs because of high moisture content and reduced grain quality. Tall wheats also have a low HI compared with short wheats. Breeding for higher grain yield with short-stemmed wheat was not very successful due to the belief that it was impossible to combine high yields with short straw (Briggle and Vogel 1968). The introduction of dwarfing genes *Rht-B1b* (formerly *Rht1)* and *Rht-D1b* (formerly *Rht2)* has been of immense importance as they were responsible for the high-yielding semi-dwarf wheats of the "Green Revolution". These genes not only reduce culm length to minimise lodging thus improving grain yield, but they have a yield advantage over tall cultivars by partitioning more assimilates to spike growth that resulted in increased yields and HI (Jain and Kulshrestha 1976). High yielding varieties carrying *Rht-B1b* or *Rht-D1b* genes also responded better to fertilizer than tall varieties (Ortiz-Monasterio R. et al. 1997).

1.2 Wheat dwarfing genes

1.2.1 *Rht-B1b, Rht-D1b* and *Rht-B1c*

There has been a general reduction in plant height over time as breeders have selected for yield and lodging resistance. Wheats with major genes for reduced height were being used in Japan in the 1800's and these were distributed to wheat breeders in the US in 1946, one line being known as Norin 10. The Norin 10 wheats were used by Dr Orville Vogel and resulted in the release of the wheat cultivar Gaines in 1961. It became a high yielding semi-dwarf wheat with reported yield increases in the Pacific Northwest of 25% (Perkins 1997) compared with the older tall wheats. Also, around this time Dr Norman Borlaug working for the Rockefeller Foundation in Mexico, had been searching for new sources of dwarfism and began using a line from Vogel named Norin10-Brevor in his breeding program. The Rockefeller Foundation's work in Mexico later formed the Centro Internacional de Mejoramiento de Maiz y Trigo (CIMMYT). Semi-dwarf wheats bred in Mexico that originated from Norin 10 formed the basis of the 'Green Revolution': a combination of breeding and management in high yielding conditions, particularly in the developing world, which resulted in greatly increased yields of wheat and rice. The new semi-dwarf varieties were all shorter than older varieties they replaced. In wheat the reduced height was due to the introduction of dwarfing genes and this was a key ingredient to the success of the 'Green Revolution' (Hedden 2003). It came about as newly bred semi-dwarf varieties of wheat and rice were able to grow with higher fertiliser inputs under irrigation without lodging before harvest. The impact of this was most evident in the 1960's in developing countries such as India and Pakistan. The genetics of the Norin 10 dwarfing genes was not established until a study by Allan (1970) on the semi-dwarf genotype. Norin 10 was found to possess both *Rht-B1b* and *Rht-D1b* alleles. These are the most important and widely used semi-dwarfing genes and are now found in over 70% of current commercial wheat cultivars globally (Evans 1998).

Rht-B1b and *Rht-D1b* were located on wheat chromosome arms 4BS and 4DS, respectively (Gale et al. 1975; Gale and Marshall 1976), and are homoeologous to each other. *Rht-B1b* was found to link with molecular marker *PSR144* and *Rht-D1b* to be linked with *GLK578* (Table 1.1). Later on, *Rht-B1b* and *Rht-D1b* was characterized by Peng et al. (1999) at the molecular level, showing that both mutations resulted from single nucleotide substitutions, and resulted in translational stop codons. Ellis et al. (2002) designed PCR-based primers targeting the single base-pair change and amplified fragments to discriminate *Rht-B1b*, *Rht-D1b* from the wild type allele.

Another dwarfing gene is *Rht-B1c* (*Rht3*), which came from the variety Tom Thumb, and this gene severely reduces the height by approx. 50% (Gale et al. 1985). *Rht-B1c* is a semi-dominant gene located on chromosome 4BS (Morris et al. 1972). *Rht-B1c* was also found to be linked with the same marker *PSR144* as *Rht-B1b* (Börner et al. 1997). The potent dwarfing gene *Rht-B1c* has a 2 kb insertion within the coding region and allele specific PCR-based markers were designed to detect the presence of the insertion in *Rht-B1c* (Pearce et al. 2011).

Table 1.1 Map location and linked markers of selected dwarfing genes in wheat (Abbreviations: RFLP, Restriction Fragment Length Polymorphism; SSR, Simple Sequence Repeats).

Dwarfing gene	Chromosome location	Linked markers	Marker type	Source	Reference
Rht-B1b	4BS	*PSR144*	RFLP	Hexaploid	(Börner et al. 1997)
Rht-D1b	4DS	*GLK578*	RFLP	Hexaploid	(Sourdille et al. 1998)
Rht-B1c	4BS	*PSR144*	RFLP	Hexaploid	(Börner et al. 1997)
Rht8	2DS	*WMS261, WMC503*	SSR	Hexaploid	(Worland and Law 1986; Ellis et al. 2005)
Rht12	5AL	*WMC410*	SSR	Hexaploid	(Korzun et al. 1997; Ellis et al. 2005)
Rht13	7BL	*WMS577*	SSR	Hexaploid	(Ellis et al. 2005)
Rht14	6AS	*BARC3*	SSR	Tetraploid	(Haque et al. 2011)
Rht16	6AS	*BARC3*	SSR	Tetraploid	(Haque et al. 2011)
Rht18	6AS	*BARC3*	SSR	Tetraploid	(Haque et al. 2011)

1.2.2 Role of GA and the mechanism of height reduction

Gibberellins (GAs) , a group of plant hormones, are essential for many developmental processes in plants, such as seed germination, stem elongation, leaf expansion, pollen maturation and induction of flowering (Achard et al. 2008). In wheat, it was first noted that *Rht-B1b, Rht-D1b* and *Rht-B1c* genotypes responded differently to exogenous GA than tall wheats. The tall wheats elongated when GA was applied to them but the dwarf wheats were insensitive (Allan et al. 1959), and these mutants were classified by Konzak (1988) as GA-insensitive dwarf mutants. Most other semi-dwarfing genes are responsive to GA, and their phenotypes are thus classified as GA-responsive dwarfs. GA was first identified in a plant fungus *Gibberella fujikuroi,* which led to exaggerated growth and lodging of infected rice plants (Yabuta 1938). There are two main groups of GA mutants discovered in plants so far, one concerned with GA biosynthesis and the other with GA signalling. The group of mutant plants that are deficient in GA biosynthesis exhibit dwarfism, but recover growth upon GA application (Reid et al. 1992). For example the mutant plant with semi-dwarfing gene *sd-1* in

rice has alterations in GA 20-oxidase gene (Os20ox2), a key enzyme to produce active GA that results in a partial block in GA biosynthesis (Monna et al. 2002; Sasaki et al. 2002; Spielmeyer et al. 2002). The other group of mutants, involving the GA signalling pathway, has been identified as GAI in *Arabidopsis* and semi-dwarfing genes such as *Rht-B1b* and *Rht-D1b* in wheat with differential response to GA.

GA-insensitive mutants such as *Rht-B1b* and *Rht-D1b* display a semi-dwarf phenotype and show a reduced response or complete insensitivity to exogenous GA. A key component of GA signalling is the DELLA protein, which acts as a negative regulator of GA response. In other words, DELLA represses growth and other GA-dependent processes, while GA releases the repressive activity (Achard and Genschik 2009). *Rht-B1b* and *Rht-D1b* in wheat were shown to be functional orthologues to *Arabidopsis* GAI and maize d8 with nucleotide substitutions in the DELLA region. Within this region, a premature translational stop codon was closely followed by several methionine initiator codons with the possibility of re-initiation to produce a truncated protein (Peng et al. 1999). The truncated protein lacks the N-terminal DELLA motif, and so it does not bind to the receptor GA complex. Thus the altered-function of the mutant DELLA protein is less affected by GA than the wild type proteins.

1.2.3 *Rht8*

Apart from GA-insensitive dwarfing genes *Rht-B1b*, *Rht-D1b* and *Rht-B1c*, there is another group of dwarfing genes that respond to GA. One of these is named as *Rht8*, which was originally from the Japanese variety Akakomugi. It was the source of reduced height for some European cultivars and was introduced by Italian breeder Strampelli in the 1920's (Worland and Law 1986). From Italy, Akakomugi dwarfism was adopted into crop breeding programs throughout Argentina before World War II, then in Europe and the former Soviet Union (Borojevic and Borojevic 2005). The genetics of Akakomugi dwarfism was characterised 70

years after its initial use in Italy and was found to co-segregate with a photoperiod-insensitivity gene called *Ppd-D1* on wheat chromosome 2D (Korzun et al. 1998). Gale (1982) located part of the Akakomugi dwarfism on 2D and it was designated as *Rht8*. Together with *Ppd-D1* gene, *Rht8* reduces plant height and changes heading date by up to eight days earlier (Gale et al. 1985).

The understanding of the mechanisms by which dwarfing genes other than *Rht-1* restrict growth in wheat is very limited. They display similar dwarfism but they are likely to have normal DELLA proteins and GA biosynthesis. Whether other growth hormones or other mechanisms account for the phenotype still needs further research. *Rht8* has a normal DELLA protein, and it was found not to be involved with defective GA-signalling but with a reduced sensitivity to brassinosteroids (Gasperini et al. 2012). The newly reported *Rht23* from an induced mutation in wheat does not alter the sensitivity of GA nor brassinosteroids and this gene may involve other hormonal pathways (Chen et al. 2014).

An important plant growth regulator that is used commercially to reduce plant height is chlorocholine chloride known as CCC. CCC inhibits GA bio-synthesis (Cao and Shannon 1997), and it is often used in high input environments, such as Europe, to reduce straw length so as to reduce lodging and increase grain yield and HI (Humphries, Welbank et al. 1965). The main purpose of this thesis is to evaluate dwarfing genes which reduce plant height in lower input environment where those plant hormones are generally not in use. The effect of CCC on wheat lines with *Rht18* is unknown.

1.3 Contributions to the grain yield and quality of conventional dwarfing genes

Increases in yield can come about from either an increase in above ground biomass or HI. In the case of the dwarfing genes all of the yield increase is due to HI as there is no penalty in above ground biomass except in extreme dwarf wheats (Austin et al. 1980a). The success of

Rht-B1b/ Rht-D1b during the Green Revolution was due to reduced height resulting in less lodging, more nutrient responsiveness and more photosynthate partitioning to the spike. *Rht-B1c* had little yield benefit due to its extremely short culm. However, its grain dormancy, an important grain quality trait was greater than tall wheats, and *Rht-B1c* has therefore been exploited in wheat breeding.

1.3.1 Grain yield improvement

In Mexico, due to the loss from lodging, the yield potential remained at 4.5 t ha^{-1} for the commercial varieties before *Rht-B1b/ Rht-D1b* was used in breeding programs. The semi-dwarf varieties broke this yield barrier and reached record yields of 8 t ha^{-1} by 1963 (Borlaug 1968). Similar improvements in yield using modern cultivars occurred in Pakistan, India, Turkey, Afghanistan and Tunisia. In Pakistan, the dwarf varieties combined with new technology for crop management were estimated to be 43% of the total harvest in 1968 (Borlaug 1968). The same year in India, 40% of the total harvest came from high yielding dwarf varieties under intensive management and the average national yield per hectare increased from 889 kg in 1967 to 1286 kg in 1968 (Borlaug 1968).

The superior yielding ability of cultivars carrying the *Rht-B1b/ Rht-D1b* genes could not be explained by lodging resistance alone. In fact the lodging resistance primarily meant that more nitrogen (N) fertiliser could be applied without the risk of lodging and this gave farmers the confidence to increase yield through higher N application. Evidence from 1966 showed that Mexican dwarf varieties Sonora 64 and Lerma Rojo 64 had progressively higher yield with the increasing rates of fertilizer applied compared with local tall variety C306 (Wright 1968). A study of the interaction between variety and fertility was conducted later at both low and high N application under irrigated conditions. Tall cultivars were compared with new semi-dwarf cultivars in Mexico, and the result showed no difference at low N treatment,

but the semi-dwarf out-yielded the tall lines when N was increased (Fischer 1981; Wall et al. 1984). Ortiz-Monasterio R. et al. (1997) confirmed that the Green Revolution semi-dwarf cultivars responded more to N than their older tall counterparts with higher N use efficiency under modern intensive management.

1.3.2 Greater ear to stem partitioning ratio

The adoption of *Rht-B1b* and *Rht-D1b* reduced plant height and lodging, and this had an effect on grain yield through a different growth pattern compared with tall wheats. Flintham et al. (1997) evaluated *Rht-B1b*, *Rht-D1b* and *Rht-B1c* in a near isogenic background in Maris Huntsman and found that the height of *Rht-B1b* or *Rht-D1b* was about 83-86% of the tall control. In comparison, both genes combined reduced height by 42%, while the severe dwarf from *Rht-B1c* caused 50% reduction (Table 1.2). This proportion can change under different cultivar background and environment. According to yield performance studies (Fischer and Wall 1976; Jain and Kulshrestha 1976; Cooper 1979), *Rht-B1b* and *Rht-D1b* were associated with increased grain number per ear and tiller number per plant, but reduced grain size especially in winter wheat (Gale et al. 1985). However, the increase in grain number and higher spikelet fertility outweighed the reduction in grain size (Gale 1979).

Studies carried out on the relationship between grain yield and plant height showed that the largest grain yield was achieved with an intermediate plant height of 60-90 cm under irrigated or rainfed conditions (Fischer and Quail 1990; Richards 1992a; Flintham et al. 1997). Grain yield was ranked for *Rht-B1b*, *Rht-D1b* and *Rht-B1c* in different experiments: Fischer and Quail (1990) reported the following ranking: *Rht-B1b* + *Rht-D1b* > *Rht-B1c* > *Rht-B1b* or *Rht-D1b* > Tall while Allan (1986) found that *Rht-B1b* = *Rht-D1b* > Tall > *Rht-B1b* + *Rht-D1b* > *Rht-B1c*. The results suggest that different dosage of *Rht-1* genes resulted in a range of plant heights at different environments, and the maximum yield can be achieved by

those plant heights that fall into the range of the optimum height. It is likely that *Rht-B1b* and *Rht-D1b* have a particular growth pattern to save assimilates for spike growth, which would otherwise be spent on stem growth.

Table 1.2 Percentages of length reductions at each internode and of changes of grain yield for dwarfing genes compared with tall controls. Length reduction results of *Rht-B1b*, *Rht-D1b* and *Rht-B1c* were extracted from Youssefian et al. (1992b) and grain yield results were calculated in cultivar Maris Huntsman from Flintham et al. (1997). Results from *Rht8* and *Rht13* were calculated from Rebetzke et al. (2011). Results from *Rht12* were calculated from Chen et al. (2013) and Rebetzke et al. (2012). Results from *Rht18* were extracted from cultivar Xifeng20 from Yang et al. (2015). (Abbreviation: Ped, peduncle; No., number)

Dwarfing gene	Plant height	Ped	P-1	P-2	P-3	Spike No./m^2	Grain yield	HI	Grain No. /spike	Grain size (mg)
Rht-B1b	-20	-22	-26	-23	-11	ns	5.9	10	13	-7.6
Rht-D1b	-20	-25	-29	-24	-14	ns	5.6	13	17	-5.8
Rht-B1c	-60	-61	-71	-67	-61	ns	-12	11	22	-17
Rht8	-8	-8	-12	ns	ns	ns	5.5	2.9	6.1	4.7
Rht12	-41	-43	-39	-41	-36	10.4^	10	19	9.3	-12
Rht13	-36	-49	-50	-38	-33	22.5	21	7.3	ns	-7
Rht18	-25	-26	-30	-28	-29	ns^	ns	11	10	-15

ns: not significant; -: reduction ^: per plant

Presence of dwarfing genes generally resulted in a greater grain number and had more fertile florets at anthesis (Siddique et al. 1989). Near-isogenic lines (NIL) carrying *Rht-B1b*, *Rht-D1b* and *Rht-B1c* were compared with tall lines and these dwarfing genes caused no effect on spikelet primordium number or timing of developmental events, while fertile florets and grain number was significantly greater in dwarfs than in the tall lines (Youssefian et al. 1992b). About 6-11 florets can be initiated at each spikelet during the primordium initiation, but most of the primordia do not go through the whole process and die before anthesis (Kirby 1974). The process that leads to floret death is not fully understood. The generally accepted

hypothesis is that floret death is due to competition between developing stems and spikes for limited resources since the photosynthetic surface is not increasing (Brooking and Kirby 1981). The competitive timing was termed as a critical period that starts with the emergence of the penultimate leaf, about 20-30 days prior to anthesis and ends at anthesis (Kirby 1988). The critical period falls within the second stage of the wheat life cycle (Figure 1.1) where the floret number is set to determine grain number as well as establish future yield. The GA insensitive dwarfing genes were associated with less competition between spike and stem growth as a greater proportion of assimilates was being partitioned to spike than stem, which led to more competent florets (Brooking and Kirby 1981; Siddique et al. 1989).

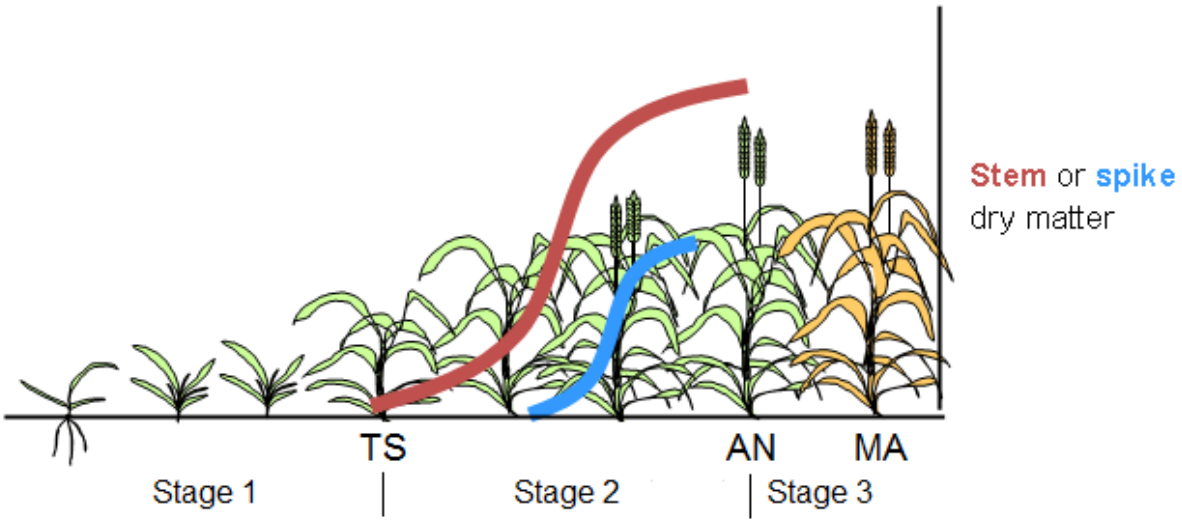


Figure 1.1 Growth stages of wheat. Stage 1 vegetative growth: from germination to terminal spikelet (TS), Stage 2 stem and spike elongation: from terminal spikelet to anthesis (AN), Stage 3 grain filling: from anthesis to maturity (MA). At Stage 1, full complements of spikelets are initiated. At Stage 2, a proportion of florets die during the differentiation and maturation. At Stage 3, dry matter from stems and leaves relocate to grain filling (Kirby 1988).

1.3.3 Post-anthesis dry matter relocation

After anthesis, wheat enters the grain filling stage (Figure 1.1) where stem elongation ceases and senescence of vegetative tissues initiates. Carbon requirements for grain filling are

derived from current photosynthate and remobilised stem reserves. The remobilisation of stored assimilates is actively translocated from stem, leaves or sheaths to grains (Zhang et al. 1998). Reduced length of internodes may have implications for water soluble carbon (WSC) storage capacity, which is an important carbon resource during the grain filling stage. WSC can reach more than 40% of stem dry weight (Blacklow et al. 1984) and up to 73% of this can be mobilised to the grain (Austin et al. 1980b). Stored WSC may account for 5-20% of the final grain yield under non-stressed condition (Austin et al. 1977), but contributes 22-60% under drought stress (Bidinger et al. 1977; Bell and Incoll 1990). The contribution of stored WSC to grain yield in wheat depended on stem storage capacity and WSC relocation efficiency (Ehdaie and Shakiba 1996). Stem storage capacity correlates with stem length and specific weight (weight per unit length) or linear density (g cm^{-1}) (Blum et al. 1994). According to Ehdaie et al. (2006), more than 50% of dry matter is stored in the basal internodes, which have more potential to store assimilate. On the other hand, the efficiency of stored WSC mobilised and translocated to grain is reported to be more related with loss of specific weight of each internode than their length (Cruz-Aguado et al. 2000). According to Borrell et al. (1993), *Rht-B1b* and *Rht-D1b* had lower estimated contribution of stored carbon to final yield than tall, with a decrease in stem mass as a percentage of the increase in grain mass. However, semi-dwarfs *Rht-B1b* exhibited greater contribution than tall in the peduncle and penultimate internodes under drought condition (Shakiba et al. 1996). Thus, the semi-dwarfing gene *Rht-B1b* or *Rht-D1b* may remobilise assimilates more efficiently than tall under non-irrigated condition.

1.3.4 Grain dormancy

The *Rht-1* genes have also been implicated in tolerance to pre-harvest sprouting. Pre-harvest sprouting (PHS) occurs when physiologically mature grain germinates in the spike before

grain harvest because of excessive moisture, e.g. following rainfall. It is one of the important factors that influences the quality of wheat grain as it reduces flour milling output and dough physical properties deteriorate (Derera 1982). PHS is a problem in many regions of the world including Asia, Oceania, Europe, North and South America. In Australia, the damage is reported to equate to $15-40 per tonne, which was about $18M AUD annually (Derera 1980). Tolerance to PHS is predominantly due to seed dormancy (Piech et al. 1970). Seed dormancy is the ability of mature seeds to avoid germinating under favourable environmental conditions. Dormancy can be affected by genes associated with seed coat colour, chemical inhibitors in vegetative tissues and spike, and seed morphological characteristics (Gfeller and Svejda 1960; King and Richards 1984; King and von Wettstein-Knowles 2000; Finch-Savage and Leubner-Metzger 2006).

PHS is associated with increased α–amylase activity that occurs with the onset of germination (Bingham and Whitmore 1966) and causes starch hydrolysis thus poor quality end-products. The α–amylase is induced by endogenous gibberellins (GA) in germinating grain (Yomo and Varner 1971). The GA-insensitive dwarf wheats had been reported to have different α–amylase level in response to increased exogenous GA treatment (Fick and Qualset 1975). *Rht-B1b* and *Rht-D1b* may reduce α–amylase activity but the result depends on genetic background and season (Gooding et al. 2012). *Rht-B1c* on the other hand has about one-fourth of the amylase activity of tall or *Rht-B1b* or *Rht-D1b* (Fick and Qualset 1975). Thus this gene provided a new genetic approach to the control of PHS (Gale and Marshall 1973; Bhatt et al. 1977). However, this gene has not been very successful in improving grain yield due to its extremely shortened plant height.

1.4 Disadvantages of conventional dwarfing genes

The 'Green Revolution' is evidence of the extraordinary success of *Rht-B1b* and *Rht-D1b*. Adoption of the new varieties occurred primarily in areas where yields were high or where irrigation water was available. Adoption of these dwarfing genes was much slower in lower yielding environments. For example the first release of semi-dwarf wheat in Eastern Australia occurred in 1973 and in 1980 in Western Australia, which was about 10 years after the release of varieties in India (Pugsley 1983). Similarly in the USA semi-dwarf wheats were only grown on 20% of the total wheat area in 1976 (Dalrymple 1980). Slow adoption of *Rht-B1b* and *Rht-D1b* had many reasons and a major one was a yield penalty under abiotic stress (Laing and Fischer 1977), such as drought and heat stress which resulted in lower grain number and grain weight (Hoogendoorn and Gale 1988; Uddin and Marshall 1989).

1.4.1 Requirements for deep sowing

A likely reason for the lower yields in adverse environment is the smaller cell size associated with wheats with *Rht-B1b/D1b* compared with tall wheats (Keyes et al. 1989). A smaller cell size reduces stem length and therefore plant height but it also reduces coleoptile length and leaf length. The latter two factors are important for emergence if sown deep, which will reduce seedling vigour.

Deep sowing (>5 cm) is an option for farmers to access soil moisture located deep in the soil profile in water limited environments. Other benefits of deep sowing include avoidance of pre-emergence herbicides (Osullivan et al. 1985), high soil temperature (Mahdi et al. 1998) and predation of germinated seeds by birds and rodents (Brown et al. 2003). However, deep sowing can have some disadvantages such as less emergence (Kirby 1993), smaller biomass and slower growth rate (Hadjichristodoulou et al. 1977; Huang and Taylor

1993) and reduced primary leaf area (Coleman et al. 2001), which can lead to a lower water-use efficiency (López-Castañeda et al. 1996) and final yield loss (Gan et al. 1992).

Under favourable moisture conditions, it is likely that both coleoptile length and early leaf area are not important. Short coleoptiles will not be disadvantageous as seeds will be planted shallow into moist soil. Reduced vigour may not be important as favourable moisture conditions will allow more time for leaf area growth and tillering. In rainfed environments, the amount of soil water found in the top soil layer is often limited at the optimum time for sowing. If they are sown too deep they will not emerge, or emerge poorly. As a result, unless sowing is delayed, wheat stands may establish poorly, resulting in lower yields (Coventry et al. 1993). This problem is exacerbated if farmers wish to sow earlier.

1.4.2 Coleoptile and early vigour

The coleoptile is a sheath that protects the developing shoot and delivers it to the soil surface. When wheat seeds are sown deep, early seedling establishment is often weak, and associated with shorter coleoptiles (Murray and Kuiper 1988; Mahdi et al. 1998; Matsui et al. 2002). A short coleoptile may expose the first leaf to hard soils, resulting in physical damage, poor stand establishment and thus a yield penalty. Crop residues or stubble can also retard plant seedling emergence. Thicker coleoptiles can help seedlings push through crusted and compacted soil by exerting greater force (Mason et al. 1994), and were found to be associated with enhanced seedling emergence in pasture species (Andrews et al. 1997). According to Rebetzke et al. (2004) coleoptile length and diameter are genetically independent, thus breeding for long and thick coleoptiles to improve seedling emergence in wheat is possible.

Coleoptile growth can be affected by a range of factors such as temperature, seed size and genetic background. Radford (1987) found that up to 70% of the reduction in coleoptile length was attributed to temperature increase from 15 to 35 °C, shortening the coleoptile by

3.8 mm/°C; thus sowing seeds into warm soil could reduce coleoptile growth and result in poor plant establishment. Seed size was another factor affecting coleoptile length; according to Cornish and Hindmarsh (1988), coleoptile length declined by 0.37 mm per mg reduction in seed weight. However, genetic backgrounds such as presence of dwarfing genes, outweighed some effects such as differences in seed position or seed source, plays an important role on determining coleoptile length (Botwright et al. 2001b).

Early vigour refers to the fast development of seedling leaf area or above-ground biomass in wheat crops (Richards and Lukacs 2002). In dry regions such as Australia, North Africa and Middle East with a Mediterranean climate, temperate cereals are planted in late autumn to early winter and harvested at the beginning of summer. Rainfall is more probable during the winter but declines prior to flowering, frequently resulting in terminal drought. Barley planted at the same time in such environments yields 20% more than wheat partly due to its better crop establishment or greater seedling vigour (López-Castañeda and Richards 1994). The greater leaf area results in more light interception and shading of ground, thus more photosynthetic assimilate and reduced evaporation from the soil leads to higher yields (Richards 1991; López-Castañeda et al. 1996). Other benefits of early vigour include higher transpiration efficiency (Bierhuiz.Jf et al. 1965), increased early root growth (Liao et al. 2004) and competition with weeds (Huel and Hucl 1996).

Traits contributing to early vigour have been identified, such as larger embryo size, fast emergence, wider first seedling leaves, high specific leaf area (leaf area to leaf weight ratio), and presence of a coleoptile tiller (López-Castañeda et al. 1995; López-Castañeda et al. 1996; Richards and Lukacs 2002). Some of these traits are correlated with each other, for example coleoptile tiller occurrence or size were positively correlated with seed size or embryo size, seedling leaf width (Rebetzke et al. 2008), as well as dry matter and leaf extension rate (Liang and Richards 1994).

1.4.3 Pleiotropic effects of dwarfing genes on seedling growth

Rht-B1b and *Rht-D1b* reduce the rate of leaf emergence and stem elongation without affecting the number of leaves or internodes (Youssefian et al. 1992a). They also improve yield and HI by producing a higher grain number with reduced grain weight. Apart from that, both genes decrease coleoptile length and early leaf area development by reduced cell size.

Allan (1989) compared Near Isogenic Lines (NILs) of *Rht-B1* and or *Rht-D1* in different backgrounds and found that lines containing *Rht-B1b, Rht-D1b* alleles have significantly shorter coleoptiles than the tall genotypes. The severe plant height reduction gene *Rht-B1c* was found to have even shorter coleoptile length compared with *Rht-B1b* or *Rht-D1b* (Addisu et al. 2009). Significant reduction of coleoptile length from *Rht-B1b* or *Rht-D1b* was confirmed and coleoptile length was positively correlated with the number of emerged plants when sown at a soil depth of 11 cm (Rebetzke et al. 2007).

Seedling leaf area is also affected. A number of studies (Richards 1992b; Rebetzke and Richards 1999) showed that *Rht-B1b*, *Rht-D1b* and *Rht-B1c* reduce seedling leaf area by decreasing leaf length and width, as the size of the epidermal cells is smaller compared with the tall genotype (Keyes et al. 1989). Miralles et al. (1998a) developed standard height, semi-dwarf and double dwarf NILs using *Rht-B1b* and *Rht-D1b* to study the effect on vegetative organs and found that except for the flag leaf, dwarfing genes reduce the cell length in vegetative organs without affecting the cell width. According to Rebetzke et al. (2001) genetic increase in coleoptile length and early leaf area in wheat populations containing *Rht-B1b* or *Rht-D1b* is limited.

1.5 Introduction of alternative dwarfing genes

This group of dwarfing genes reduce wheat culm length, improve lodging resistance, and respond to exogenous GA. They were discovered as spontaneous variants or through

mutagenesis in durum and bread wheat and studies showed that some of them do not compromise coleoptile length or seedling leaf area but still increase grain yield. Thus these dwarfing genes could potentially replace GA-insensitive genes *Rht-B1b* or *Rht-D1b* in water-limited regions. These alternative dwarfing genes include *Rht8, Rht12, Rht13, Rht14, Rht16* and *Rht18*.

1.5.1 History and map location

In southern Europe where high temperatures occur around the time of meiosis, *Rht-B1b* and *Rht-D1b* interacted with the environment and caused fertility reductions and loss of yield (Law and Worland 1985). The germplasm derived from the Japanese variety Akakomugi which contained *Rht8* and was well-adapted to southern European environments with no reduction in fertility under high temperature (Worland and Law 1986). The height reducing phenotype of *Rht8* reduced height by 7-8 cm in England and the former Yugoslavia compared with tall lines without detriment to other agronomic traits (Worland et al. 1998). In the vegetative organs, *Rht8* reduces each internode by decreasing cell elongation in the stem (Gasperini et al. 2012), while the cell length and width of leaf cells (leaf 2) were not affected by this allele, suggesting the plant height is independent from leaf cell dimension (Botwright et al. 2005). *Rht8* was described as a weak allele for height reduction and mapped to the short arm of chromosome 2D (Worland et al. 1990) and closely linked to SSR marker gwm261 about 0.6cM distal to the gene (Korzun et al. 1998). The 192 bp allele amplified from gwm261 was associated with height reduction (Worland et al. 1998). However, the diagnostic 192 bp allele is also present in cultivars that do not carry *Rht8*, which limited its application in breeding programs. Later Gasperini et al. (2012) developed gene based markers to fine map *Rht8*.

Rht12 is a dominant GA-responsive dwarfing gene, derived from gamma ray-induced mutagenesis, released as ‘Karcagi 522m7K’ from the bread wheat variety ‘Karcag 522’.

Rht12 is known to have a strong reduction in height (approx. 37-40%), accompanied by thicker internodes that may contribute to lodging resistance (Chen et al. 2013). *Rht12* was not successful commercially due to its height reduction being too great, reduced grain size and delayed ear emergence (Worland et al. 1994). However, *Rht12* was recently found to increase yield and HI with an increased grain number per spike or tiller number per unit area (Rebetzke et al. 2012; Chen et al. 2013). *Rht12* was previously mapped to the distal region of chromosome 5 AL (Table 1.1).

Rht13 was first discovered as a semi-dominant, GA responsive gene in gamma-ray induced mutant derived from bread wheat variety Magnif 41. The height reduction ranges from 45% to 50%, but it was not evident until ear emergence (Rebetzke et al. 2011). In other words, the reduction in *Rht13* mainly takes place in the peduncle and penultimate internodes at a time when carbon resources for floret development and fertility are most needed. According to Rebetzke et al. (2011), *Rht13* was also associated with increased grain number, yield and HI. *Rht13* was mapped on chromosome 7BL and linked with SSR marker *WMS577* (Ellis et al. 2005).

GA-responsive gene *Rht14* from mutant CpB132 later named Castelporziano was a semi-dwarf, produced by thermal neutron treatment from tall Italian durum variety Cappelli. Castelporziano was developed in Italy in 1956 to increase the yield of durum wheat with a focus on lodging resistance. Between 1968 and 1971, two cultivars were released as direct selections from Cappelli named cv. Castelfusano and cv. Castelporziano. *Rht14* is a semi-dominant gene that reduces plant height by 34% of the variety Cappelli (Gale et al. 1985). This gene has been found to have positive effects on yield through increased tiller number per plant (Gale et al. 1985).

Rht16 is another GA-responsive gene generated by treating spring durum variety Edmore with methylnitrosourea and released as Edmore M1(Konzak 1987). Genetic studies

of this gene by Konzak (1988) indicated that *Rht16* was a semi-dominant gene, and it reduced height by 30% relative to Edmore.

Rht18 was generated in durum wheat through mutagenesis on tall cultivar Anhinga using fast neutron radiation. A semi-dwarf mutant was identified and released as cultivar "Icaro" which carried a single semi-dwarfing gene named *Rht18* in 1987 (Konzak 1987). The physiological characterisation of this gene has been reported in durum and bread wheat. Plant height was reduced about 30% evenly across all internodes, without affecting yield but increasing HI due to the reduction in stem weight in durum wheat (Maddocks 2008). A recent publication found *Rht18* reduced plant height moderately by 18% on average without affecting grain yield in three Chinese bread wheat cultivars (Yang et al. 2015).

Rht14, Rht16, and *Rht18* were mapped in tetraploid wheat and linked to SSR marker *BARC3* on chromosome 6AS (Table 1.1). *Rht14, Rht16* and *Rht18* were independently generated following mutagenesis in different durum cultivars, and Haque et al. (2011) suggested that these three genes could be allelic since no tall lines were recovered in F_2 progenies derived from crosses between Castelporziano, Edmore M1 and Icaro.

1.5.2 Coleoptile length and early seedling growth in wheat with alternative dwarfing genes

Many alternative dwarfing genes reduced plant height without compromising seedling growth traits, and thus have the potential to replace *Rht-B1b* and *Rht-D1b*. According to Rebetzke and Richards (2000a) and Ellis et al. (2004), coleoptile length and seedling leaf area were not affected by *Rht8,* and coleoptile length is independent of the height reduction, thus breeding aimed to combine long coleoptile with reduced height wheat (Rebetzke et al. 1999). *Rht12* showed no effect on coleoptile length and seedling leaf area, thus it is possible to replace *Rht-B1b* and *Rht-D1b* in autumn sowing environment (Chen et al. 2013). Ellis et al. (2004)

compared the coleoptile length between wild type and mutant Magnif (*Rht13*) and found no significant differences, suggesting *Rht13* is a good alternative to *Rht-B1b* or *Rht-D1b.* Also, *Rht14* and *Rht16* had no effect on coleoptile length thus have potential in breeding (Konzak 1987; Konzak 1988). The coleoptile length of *Rht18* was studied by Ellis et al. (2004) comparing the mutant Icaro with wild type Anhinga, and the result showed that Icaro had significantly shorter coleoptiles than Anhinga, suggesting that *Rht18* reduces coleoptile length. However, the study may have been confounded by background mutations induced by random mutagenesis to reduce coleoptile length in Icaro, but not in wild type Anhinga. Yang et al. (2015) compared coleoptile length between *Rht18* lines and three bread wheat cultivars which were used to develop populations segregating for the *Rht18* lines. The result showed no difference between dwarf lines and tall cultivars from two populations, we therefore concluded that *Rht18* is unlikely to reduce coleoptile length.

1.5.3 Height and grain yield

Dwarfing genes reduce plant height, which may not be translated into greater HI and yield. Grain yield can be affected by growth patterns before and after anthesis, especially the period when stem and ear grow rapidly pre-anthesis and dry matter relocates post-anthesis. Relatively little is known about the effect of alternative dwarfing genes on growth and yield compared with conventional ones.

Similar to *Rht-B1b* and *Rht-D1b*, the alternative dwarfing genes generally reduce the length of all internodes, rather than being concentrated in a particular one (Table 1.2). Nearly all the dwarfing genes described in this review have a proportional reduction in internode length. An exception is *Rht13* where the peduncle and P-1 internodes are relatively shorter than the basal internodes.

As previously mentioned, optimum grain yield can be achieved with intermediate height (60-90 cm) in GA-insensitive dwarfing lines under both irrigated and rainfed conditions (Chapter 1.3.2). Similarly, an intermediate height also leads to optimum grain yield for lines with GA responsive alternative dwarfing genes (Table 1.2) such as *Rht13*, which has contributed more fertile tillers per unit area or per plant. All listed dwarfing genes increased HI while reducing plant height such as *Rht12*, which shortens each internode by approx. 40%.

The reduction in plant height caused by alternative dwarfing genes is also correlated with increased grain number per spike to boost grain yield as with conventional dwarfing genes (Table 1.2). *Rht8, Rht12* and *Rht13* showed increased grain number per spike (or per m^2) and may have a similar pattern of assimilate distribution to the spike. For example, higher grain number was found in NIL lines carrying GA sensitive gene *Rht13*, which shortens the peduncle and penultimate internode, allowing more assimilates to be partitioned to grain (Rebetzke et al. 2011). Increased grain number is often associated with decreases in grain size (weight per kernel) in the listed alternative dwarfing genes. *Rht18* was reported to have more grains along with smaller grain size. *Rht12* has a stronger dwarfing effect, resulting in higher grain numbers per spike and smaller grain. Fortunately, the increased grain number outweighed a decrease in grain size resulting in grain yield advantage for *Rht8, Rht12* and *Rht13*.

The contribution of dry matter from stem and leaves to grain development post-anthesis has been reported for *Rht12*. The results suggest that *Rht12* relocates less dry matter to grain growth, has a smaller gain in grain weight (Chen et al. 2013) and has similar α–amylase activity compared with the tall in near-isogenic background (Gooding et al. 2012). More and detailed studies for physiological traits of alternative dwarfing genes are needed in order to evaluate their replacement for conventional dwarfing genes.

1.6 Mapping and marker development

1.6.1 Using molecular markers to identify physiological traits

Conventional plant breeding is largely dependent on the selection of desirable traits, which are likely to be affected by the interactions of genetic and environmental factors. Plant breeders usually select traits such as high yield and disease resistance by crossing plants with those desirable traits and selecting superior progeny under different environments, which can take 10-15 years. Molecular markers may shorten the breeding process and their use is termed marker-assisted selection (MAS) (Beckmann and Soller 1986). MAS involves selecting individual plants based on the DNA pattern rather than observable traits and it has distinct advantages compared with conventional breeding processes. First, MAS can be applied to the seedling material thus reducing the time required to demonstrate the presence of a particular trait. For example, some traits are only observable at flowering or maturity, so genotyping the plant before flowering could allow key steps such as crossing without needing to wait till the next generation. Second, MAS is not affected by environment. Abiotic stress or disease resistance are usually evaluated across different years, but with the help of molecular markers for these facts, plant resistance levels can be determined independently of environment. Third, recessive alleles can be identified by molecular markers in the presence of dominant alleles. In conventional breeding programs, recessive alleles can only be identified through progeny testing, while MAS can be used to identify recessive alleles using linked markers without the need for additional generations. Fourth, gene pyramiding can easily be implemented by molecular markers. For instance, when multiple genes are combined in the same line or variety, the presence of each gene is difficult to verify phenotypically. With individual genes tagged by different markers, this problem can be solved.

1.6.2 Determining the map location is required for development of tightly linked markers

MAS has some limitations. One of the key disadvantages of this technique is recombination between the marker and the gene/trait, which could lead to false positives. Thus tightly linking the marker to the trait is desirable for successful MAS. In order to do this, the first step is to find the chromosome location of a trait and construct a linkage map that provides a framework of marker-trait association. Once a marker is identified as associated with a trait, closer markers can be developed. Bulk Segregant Analysis (BSA) can rapidly identify markers in a genomic region linked to a trait (Michelmore et al. 1991). BSA partitions families from a single cross into two phenotypically opposite bulks (genomic DNA) according to a trait and measures the correlation between markers and phenotype samples from each bulk thus designating a probable location that is associated with the markers. Another method called selective genotyping analyses selected families from two extreme groups individually rather than pooled (Darvasi and Soller 1992), and therefore it provides better precision and certainty for identification of map locations (Sun et al. 2010).

Molecular markers are classified based on their detection method and throughput. Simple Sequence Repeat (SSR) or microsatellites is a PCR-based, medium throughput marker. They were declared the ‘markers of choice’ after its discovery due to their high abundance and level of polymorphism (Powell et al. 1996). The application of SSR markers has been aided by high-throughput genotyping platforms such as capillary electrophoresis allowing separation of PCR products from multiple markers at the same time and with high resolution (Lu et al. 1994; Wenz et al. 1998). A high-density SSR map for bread wheat was constructed using markers from different research groups and consisting of 1,235 microsatellite loci, covering 2,569 cM, with an average interval distance of 2.2cM (Somers et

al. 2004). This work had helped to tag many genes of economic importance in wheat (William et al. 2007).

Single Nucleotide Polymorphisms (SNPs) are less polymorphic than SSR markers due to their bi-allelic nature, yet their abundance and amenability to easy automation has meant that this technology has largely replaced the use of SSR markers. Conventional SNP discovery was associated with low frequency and high cost (Batley et al. 2003; Wright et al. 2005), but the recent emergence of Next-Generation Sequencing (NGS) technologies such as transcriptome re-sequencing has overcome the hurdles of low throughput and high cost of SNP discovery (Morozova and Marra 2008). However, transcriptome re-sequencing targeting coding regions will not identify SNPs located on non-coding regions. The presence of large regions of repetitive and duplicated DNA is also problematic. The advent of Complexity Reduction of Polymorphic Sequences (CRoPS) (van Orsouw et al. 2007) and Restriction Site Associated DNA (RAD) (Baird et al. 2008) has made the genome-wide high throughput SNP discovery possible by filtering out repetitive SNPs. These techniques coupled with NGS have facilitated many genome-wide studies (Elshire et al. 2011; Poland et al. 2012). Discovered SNPs were used to construct 9K and 90K arrays to facilitate high-throughput genotyping. In wheat, a 9K gene-associated SNP array was constructed to assess genetic variation of 2,994 wheat accessions (Cavanagh et al. 2013). The recent 90K SNP array which includes a significant fraction of common genome-wide distributed SNPs from both allohexaploid and allotretraploid wheat populations of diverse geographical origin will serve as an invaluable source for SNPs linkage to important traits in wheat (Wang et al. 2014). The large number of SNP markers from various germplasm can be used to achieve dense and high-resolution mapping of the genome. A high density consensus map was constructed integrating both SNP and SSR markers from different mapping populations for A and B genomes in durum wheat (Marone et al. 2012), and the linkage map including DArT, SSR and SNP markers in bread

wheat (Huang et al. 2012) allowed analysis of gene-trait association for agronomical important wheat phenotypes.

1.6.3 Converting tightly linked markers into robust assays

The SNP polymorphisms need to be converted to robust markers before they can be genotyped. The validation of a marker is the process of designing assays based on the discovered polymorphism and testing the marker within the experimental population. The most popular platform to assay SNP markers is Kompetitive Allele Specific PCR (KASP^{TM}). This platform is a PCR-based protocol and is designed to distinguish between alleles that differ only by a SNP. KASP SNP genotyping is based on allele-specific amplification and Fluorescence Resonance Energy Transfer (FRET). Each assay consists of three primers that include two allele-specific and a common primer. The two allele-specific primers have their 3'ends complementary to each of the SNP allele and their 5' ends are designed to bind with a different fluorophores mixed in the reaction agent. During PCR reaction, fluorescent oligonucleotides are incorporated in the product and alternative SNPs can be discriminated by different colours via a plate reader (http://www.lgcgenomics.com).

The first step in marker validation is to confirm polymorphism between the parents of the experimental population that segregate for the trait. When the marker is polymorphic between the parents, it is then assayed in the population and the strength of association between the marker genotype and the phenotype is evaluated. Significant association indicates the potential utility of the marker in breeding. Even though the marker is confirmed to be effective in the mapping population, it still need to be tested in a wider range of germplasm which includes a panel of cultivars or breeding material with reliable phenotypic data.

1.6.4 Evaluating markers to determine the frequency of the allele

Markers developed from experimental populations require evaluation across a wide range of germplasm to determine the frequency of the allele before their utility in breeding can be assessed. The target allele will only be useful to breeders when the frequency is low in backgrounds that lack the trait.

Available SNPs from arrays may not be present in the experimental populations even though the populations were sourced from wide range of germplasm. When available SNPs are not sufficient to develop markers which can detect low frequency target alleles, other approaches are required to discover more SNPs. NGS technology provides genome wide SNP discovery in a single sequencing step. Accompanied by a restriction enzymes step, this technology has developed markers based on sequenced SNP or structural variants and became an extremely versatile and cost-effective assay. NGS can produce tens of thousands of markers with high genotyping accuracy, which can aid SNP discovery in different experimental populations.

Genome sequencing is the foundation to understand the molecular basis of phenotypic variation, and it relies on the building of physical maps for high quality sequence assembly. Unlike the genetic map presented by markers with genetic distance which is measured by recombination of frequency between those markers, the physical map shows the actual locations of genes or DNA sequences of interest separated by base-pair distances. Physical maps are constructed by shearing the genome into smaller pieces that are cloned and stored as large DNA fragment libraries in Bacterial Artificial Chromosomes (BAC). A set of overlapping DNA fragments that are contained in BACs are called contigs, and this can be assembled to determine the sequence of the targeted genome. Physical contigs can be anchored onto a genetic map with markers and the clones can then be sequenced to help identify new markers or to characterise the region. Physical maps and whole genome

sequence have been generated for some cereals such as sorghum, rice and maize (Klein et al. 2000; Chen et al. 2002; Wei et al. 2007). International collaboration on wheat genome sequencing was initiated in 2003 with the aim to develop 21 chromosome maps by chromosome-specific BAC libraries (Gill et al. 2004). The wheat cultivar Chinese Spring was chosen as the reference for genomic sequencing by IWGSC because of its large genetic stock of aneuploid lines (Endo and Gill 1996). The bread wheat physical map has 10-fold coverage of the 17 Gb genome size that requires more than 1.4 million BAC clones to be assembled into contigs and anchored to the genetic map. Paux et al. (2008) constructed a BAC-based integrated physical map on the largest chromosome 3B (995 Mb). Recently the 6A physical map has been assembled and anchored with 1217 contigs for the short arm and 1113 contigs for the long arm and 79% of the physical map anchored to genetic map (Poursarebani et al. 2014). The sequence information from 6A will help to discover SNP markers for *Rht18* which has been identified on chromosome 6AS.

1.6.5 Development of high-throughput assays for breeders to assist in selection

A useful marker must be tightly linked to a target trait and it should be robust, low cost, high throughput, low volume and diagnostic across breeding germplasm. Once a robust marker is developed with a low frequency of target allele, marker information can be used to develop high-throughput assays to assist breeders in breeding programs. Due to the vast numbers of materials that need to be genotyped, automation of genotyping that combines automation of sample preparation with analysis has played a key role in the breeding programs (Dayteg et al. 2007). SNP marker is preferred due to the automation of SNP genotyping platform than other markers such as SSRs. Commercial automation of SNP genotyping platform is available such as TaqMan SNP genotyping assay which can be performed using an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). It is a high

throughput genotyping platform which is suitable for screening large number of lines on selected SNP markers during the breeding process.

1.7 Objectives of this study

This study focuses on *Rht18* as one of the alternative dwarfing genes in wheat to better understand its effects on growth and yield with the possibility of replacing *Rht-B1b* and *Rht-D1b* under rainfed environments. The study consists of two aspects, one is physiological and the other is genetic. Germplasm was developed in this study so that direct comparisons could made between *Rht18*, *Rht-D1b/B1b*, Tall (*Rht-D1a/B1a*) and Double dwarf (*Rht18+Rht-D1b/B1b*). Morphological measurements concentrated on seed and seedling vigour traits such as coleoptile length, seedling emergence from deep sowing, early leaf area and grain dormancy; yield related traits such as height reducing effect, stem and spike growth, grain yield and HI were undertaken by comparing *Rht18* to *Rht-D1b* or *Rht-B1b*. The genetic study of *Rht18* aimed to develop molecular markers, especially easy to use SNP markers, to assist in breeding. Apart from the fine mapping of *Rht18*, this study also investigated allelism of *Rht18, Rht14* and *Rht16* and the map locations of *Rht14* and *Rht16* indicated by Haque et al. (2011). The recent study of *Rht18* in bread wheat (Yang et al. 2015) was published just prior to the completion of this thesis, and the study covers some overlapping interests such as height, grain yield and coleoptile length, which will be discussed in the appropriate chapters.

Chapter 2 Effect of *Rht18* on height, flowering time and yield in bread wheat

2.1 Introduction

This chapter examines the impact of the reduced height gene *Rht18* on final height, internode length, flowering time and grain yield in a set of related wheat lines.

Yield improvement from the height reducing genes *Rht-B1b* and *Rht-D1b* is not only due to reduced susceptibility to lodging but also to more assimilate partitioned to the spike than to stem growth at anthesis (flowering time) (Brooking and Kirby 1981), and higher HI at maturity (Jain and Kulshrestha 1976). Height reduction by *Rht-B1b* or *Rht-D1b* is due to insensitivity of the cells responsible for extension growth in the stem to the plant hormone gibberellin, and hence shorter stem length as a result of reduced cell size (Keyes et al. 1989; Miralles et al. 1998a). The reduction in plant height in *Rht-B1b* and *Rht-D1b* was also associated with more fertile florets at anthesis, which in turn increased grain number. This is believed to be a pleiotropic effect of Norin 10 derived dwarfing genes (Siddique et al. 1989; Youssefian et al. 1992a), which could come about through the height reducing genes allocating more assimilates to the developing spikes than to the stem during the period of stem elongation (Fischer and Stockman 1980; Kirby 1988). Reducing the peduncle length had been proposed as a way to increase grain number and yield in wheat (Richards 1996) and new dwarfing genes offers the potential to do this (Rebetzke et al. 2011).

In contrast to *Rht-B1b* or *Rht-D1b*, other dwarfing genes exist, such as *Rht8*, *Rht12* and *Rht13* shown in Table 1.1 Chapter 1, and they are sensitive to gibberellin. Thus, stem internode length reduction can also be achieved by different *Rht* genes, but the dwarfing mechanisms responsible for the GA-responsive dwarfing genes remain unknown.

The timing of phenological development is also an important trait as this is associated with management decisions and grain yield (Biscoe and Wellington 1984). According to Youssefian et al. (1992a) *Rht-B1b* and *Rht-D1b* only change the rate of leaf emergence or stem elongation without affecting the timing of developmental events or number of leaves or internodes. For example, Fischer and Quail (1990) reported no difference in time of anthesis for *Rht-B1b*, *Rht-D1b*, *Rht-B1c* and Tall genotypes. However, plant height in different environments may be variable. Richards (1992a) compared flowering time in a set of isogenic lines for *Rht-B1b*, *Rht-D1b*, *Rht-B1c* and *Rht-B1b+Rht-D1b* with Tall. The results showed that tall lines without the dwarfing gene flowered one and a half days earlier than lines with a single dwarfing gene, and the single dwarfing gene lines flowered about one day earlier than double dwarfing genes. Similar results are reported by Fischer and Stockman (1986), where dwarfing genes had a slightly longer duration from initiation to anthesis than the tall wheats. As for GA-responsive genes, *Rht12* has been reported to delay ear emergence (Worland et al. 1994), and Castelporziano (*Rht14*) was reported to be 2-3 days later than the wild type cultivar Cappelli (Bozzini 1974). Other GA-responsive dwarfing genes were not reported to have a significant effect on anthesis date.

Experiments in this chapter were undertaken to establish the effect of height reducing genotypes in Espada and Young backgrounds, to compare standard semi-dwarfs with *Rht18* by examining plant height, internode length and grain yield.

2.2 Materials and methods

2.2.1 Plant material and cultivation

The populations used in these experiments are listed in Table 2.1. All populations were developed from a cross between Espada or Young, elite Australian bread wheat cultivars carrying *Rht-D1b* or *Rht-B1b* and the bread wheat *Rht18* donor line HI25M (courtesy of Greg

Rebetzke and Allan Rattey CSIRO), which was derived from a cross between Icaro (tetraploid, *Rht18*) and Halberd (hexaploid). Genotyping information for populations is given in Table 2.2. In 2012, F_5 families (Expt 1) with each line derived from a single F_2 plant from the biparental cross HI25M (*Rht18*) × Espada were sown in rows at Black Mountain, Canberra (Latitude: -35º 16", Longitude: 149º 6" E) and 13 sequential harvests of main stems from five plants were taken from terminal spikelet (TS) to maturity. Main stem was classified as the tallest stem from a plant. In 2013 two populations were sown, F_6 families (Expt 2) were sown in plots at Ginninderra Experiment Station (GES), Canberra (Elevation 600m, Latitude: -35º 12", Longitude: 149º 4" E). Quadrats were harvested at physiological maturity and plot harvests were made with a machine harvester (Dominator, CLAAS). BC_2F_4 families with each line derived from a single BC_2F_2 plant (Expt 3) were sown in rows at Black Mountain and harvested at 15 sampling times. The main stems from five plants were harvested from TS to maturity. In 2014 three populations were sown, BC_2F_5 (Expt 4) at GES in plots and five tillers were harvested at three sampling times (10 days before anthesis, 7 days after anthesis and 28 days after anthesis). BC_2F_4 families (Expt 5) from HI25M × Espada and BC_2F_4 (Expt 6) from HI25M × Young (*Rht-B1b*) were sown in rows at GES and five plants per row were harvested at 14 days after anthesis. There were four genotypes in every experiment (Table 2.2) and there were five lines per genotype. Each line was chosen after genotyping (see Section 2.2.2). Lines in all experiments have two replicates and lines in Expt 4, Expt 5 and Expt 6 were randomised. Plants grown at Black Mountain were irrigated while GES was rainfed.

Table 2.1 Populations deployed in growth and yield studies with sowing dates

ID	Population	Parent 1	Parent 2/ Recurrent	Sowing pattern	Spike and stem growth	Grain yield
Expt 1	F_5	HI25M	Espada	Row	18th Sep 2012	
Expt 2	F_6	HI25M	Espada	Plot		24th May 2013
Expt 3	BC_2F_4	HI25M	Espada	Row	15th May 2013	
Expt 4	BC_2F_5	HI25M	Espada	Plot	19th June 2014	19th June 2014
Expt 5	BC_2F_4	HI25M	Espada	Row	20th June 2014	
Expt 6	BC_2F_4	HI25M	Young	Row	20th June 2014	

Experiments at Black Mountain were sown in single rows (12 meters long) spaced by 30 cm with Granulock15 (N, P, S of 14.3, 12, 10.5) at a rate of 110 kg/ha and top dressed with 80 kg/ha urea at booting stage. Expt 1 and 3 at Black Mountain were irrigated when the soil was dry. Plots at GES were sown using an Agrowdrill (Agrowplow) with 50 g seed per plot with a 15 cm between-row spacing, and 2-5 cm within row spacing. Plots were 6 m long and 10 rows wide and the quadrats were 1.2 m long and 0.3 m wide. Fertiliser was applied at sowing (Granulock 15) at 110kg/ha. The soil type in Expt 2 and Expt 4 at GES were grey brown clay (Alluvial flats) and shallow red podzolic soil respectively. The row tests Ext 5 and 6 in GES were arranged in the same way as those in Black Mountain but cultivated in the same way as plots. Plots were not irrigated and the temperature and rainfall data were extracted from GES Automatic Weather Station and listed in Appendix Table 2.1. Weather data from GES were also applicable to Black Mountain, except that temperature used to calculate thermal time in Expt 1 and Expt 3 was from a temperature logger placed at the site.

Expt 4, 5 and 6 sown in plots and rows at GES were severely damaged by birds at physiological maturity. Plot harvests were not made and only limited data could be collected.

2.2.2 Genotyping

Families from HI25M crossed with Espada or Young were genotyped with markers for *Rht-D1b* or *Rht-B1b* designed by Ellis et al. (2002). *Rht18* is tightly linked with SSR marker *WMS4603* (Spielmeyer et al. unpublished), and lines with *Rht18* carried the 239 bp allele in contrast with 220 bp in lines lacking *Rht18*. All lines used in phenotypic experiments were fixed according to the Table 2.2.

Table 2.2 Pedigree information in populations with four genotypic classes developed from HI25M and Espada (*Rht-D1b*) or Young (*Rht-B1b*)

Genotype	*Rht-1*	*Rht18*
Rht18	*Rht-D1a/B1a*	*Rht18*
Rht-D1b/B1b	*Rht-D1b/B1b*	-
Tall	*Rht-D1a/B1a*	-
Double dwarf	*Rht-D1b/B1b*	*Rht18*

2.2.3 Morphological measurements

Plant height was measured from the soil surface to the tip of the spike. Plant height and internode length were measured using a ruler and recorded in millimetres. The whole stem (free from leaf sheath) was dissected into different internodes and named in order from top to bottom as: Peduncle, P-1, P-2 and P-3 as shown in Figure 2.1.

Flowering time was determined using the Zadok's scale in all lines in Expt 1 and 3 when around 50% or more of ears were flowering (anthers visible) and 7 days past first flowering in Expt 2 and 4.

Grain weight, grain number, biomass and yield were measured after threshing (Wheat Head Thresher, Model: WHTA010002 220v, Precision Machine Co., Inc.), and grain number was calculated using a seed counter (Contador, PFEUFFER GmbH). Biomass was determined

for the dried plants, and harvest index was calculated as the ratio of grain weight to biomass. Yield in the plots was recorded as grain weight during final machine harvest.

2.2.4 Experiment design and data analysis

Randomisation was arranged at the line level across four genotypes in the experiments conducted at Black Mountain and GES. Statistical analysis was performed for the effect of genotype using ANOVA in Genstat (V16th Edition) and the l.s.d. was provided. In Expt 2, where no genotypic difference was observed, contrast comparison model in ANOVA in Genstat was also used to look at chosen sets of comparisons individually.

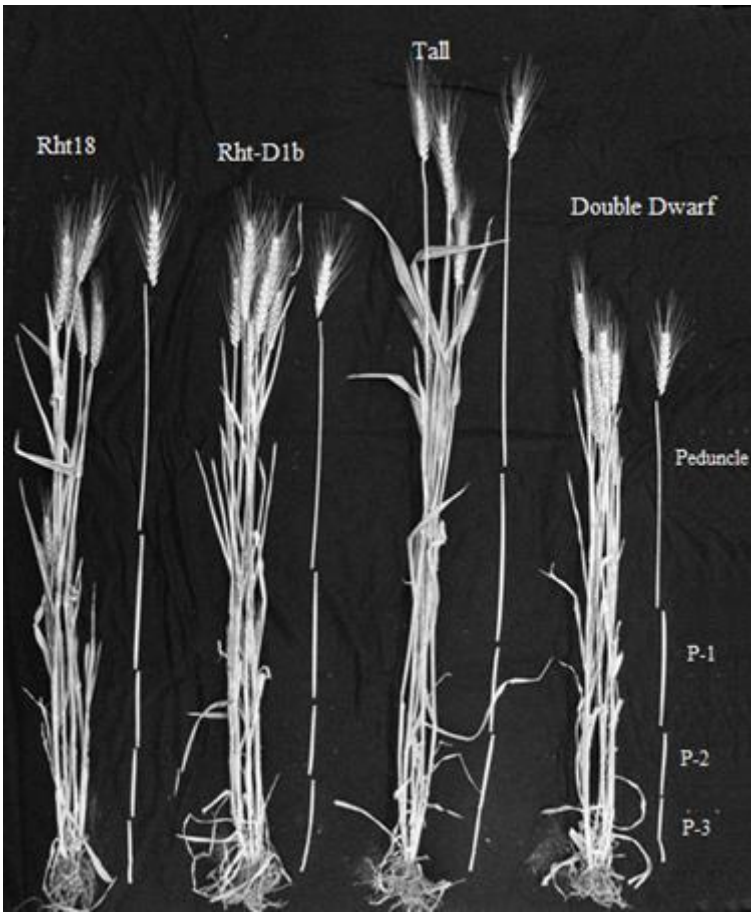


Figure 2.1 Stem was dissected into 4 sections recorded as peduncle, P-1, P-2 and P-3+ (includes the lower internodes) in 4 genotypic classes: *Rht18*, *Rht-D1b*, Tall and Double dwarf.

2.3 Results

2.3.1 Final height and internode length

Five populations were derived from two crosses and each population segregated for two semi-dwarfing genes *Rht18* and *Rht-D1b/B1b*. Results were generally consistent across all environments (Table 2.3).

According to weather data from Appendix Table 2.1, seasonal mean (growth period) temperatures in both minimum and maximum were a bit lower than long term mean (averaged over the year) temperatures in Year 2013 and 2014, since the sowing in Year 2012 was later than others. Minimum and maximum temperatures during growth periods were similar between Year 2013 and Year 2014. Rainfall in 2012 was more than the other two years, but this not apparent in the growth season.

Table 2.3 Means of final height (cm) (including spike length) for different genotypes in different experiments. (Abbreviation: *D1b/B1b*, *Rht-D1b/B1b*; DD, Double dwarf)

Genotype	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 6	Average
Rht18	57.8	67.7	82.7	67.2	60.1	57.6	65.5
D1b/B1b	57.4	59.5	81.2	67.5	63.5	57.1	64.4
Tall	87.5	95.0	103.4	87.1	77.3	75.2	87.5
DD	48.8	56.6	74.2	60.3	56.1	49.7	57.6
Average	62.7	69.5	85.2	70.3	64.1	59.9	

The l.s.d. was 2.4 (Expt***), 2.0 (Genotype***), and 4.8 (Expt × Genotype***). ***: $P<0.001$

There was no significant difference in plant height between *Rht18* and *Rht-D1b/B1b* in all experiments except Expt 2, suggesting both genes reduce plant height by the same amount across environments. In Expt 2, *Rht18* was taller than *Rht-D1b* by approx. 8 cm, which may be due to some heterozygous background genes fixed in this generation compared with its earlier generation in Expt 1. Averaged over all experiments *Rht18* was 23% shorter than Tall lines and 11% taller than Double dwarf lines. Variation in plant height was attributed to an

interaction between genotype and environment (Table 2.4). For example, sowing earlier tended to result in taller plants (Expt 3 sown in May 2013, Table 2.3) than when planted later (Expt1). Genetic background also affected plant height (Expt 5 and Expt 6).

Table 2.4 Summary of significance of the main effects and interaction between genotype and environment for distal three internode lengths and percentages of each internode to total stem in Expt 1, 3, 4, 5, 6.

Treatment	Total stem (cm)	Peduncle (cm)	Peduncle %	P-1 (cm)	P-1%	P-2 (cm)	P-2%
Genotype	***	***	*	***	ns	***	***
Experiment	***	***	***	***	***	***	***
G×E	***	***	ns	*	*	*	ns

*: $P<0.05$, ***: $P<0.001$, ns: not significant

Table 2.5 Means of distal three internode lengths and percentages of each internode to total stem in Expt 1, 3, 4, 5, 6. (Abbreviation: *D1b/B1b*, *Rht-D1b/B1b*; DD, Double dwarf)

Genotype	Total stem (cm)	Peduncle (cm)	Peduncle %	P-1 (cm)	P-1%	P-2 (cm)	P-2%
Rht18	56.1	27.4	49.4	13.4	23.9	8.2	14.5
D1b/B1b	56.6	27.2	48.5	14.0	24.6	8.2	14.6
Tall	77.2	38.5	50.1	19.0	24.7	10.4	13.5
DD	49.1	23.1	47.5	12.2	24.7	7.6	15.3
l.s.d.	2.1***	1.1***	1.7*	0.7***	ns	5.6***	0.7***

*: $P<0.05$, ***: $P<0.001$, ns: not significant

Distal three internodes of *Rht18* and *Rht-D1b* were almost identical in length. Internodes were longest in the Tall and shortest in the Double dwarf. When each internode length was expressed as a percentage of the total stem length, there was little difference between any of the genotypes (Table 2.5). However, the difference in height between Tall and Double dwarf in peduncle percentage, and between Tall and other dwarfing genes in P-2 percentage, suggest a slight difference associated with *Rht18*, *Rht-D1b/B1b* and Double dwarf

compared with tall lines. Internode lengths and percentages were displayed for four genotypes in individual environment (Expt 1, 3, 4, 5, 6 in Appendix Figure 2.1).

2.3.2 Flowering time

Developmental stages were recorded using the Zadoks scale for the four experiments post anthesis (Table 2.6). All genotypes flowered at the same time in each of the four experiments, thus, there was no evidence that either *Rht-D1b* or *Rht18* affected flowering time.

Table 2.6 Zadoks score of four genotypes in four experiments

Population	Expt 1	Expt 2	Expt 3	Expt 4
Rht18	63.5±0.4	70±0.3	61.6±0.2	69.4±0.2
Rht-D1b	62.7±0.5	69±0.3	62.2±0.4	70.2±0.2
Tall	63.0±0.4	70±0.3	62.0±0.5	69.4±0.2
Double dwarf	61.5±0.5	70±0.2	61.2±0.6	69.6±0.4

2.3.3 Grain yield and harvest index

Grain yield and related traits of the four genotypes were examined for the main stem, plant and per unit area to address whether *Rht18* has any yield penalty compared with *Rht-D1b* and tall lines.

Data from Expt 1, 2 and 4 were based on main stem yield (Table 2.7). In Expt 3, yield and its components were measured on a whole plant basis (Table 2.8), and in Expt 2, biomass, grain yield and HI was measured at quadrat or plot level and reported as per unit area (Table 2.9). Different experiments had highly significant differences in all traits except HI (Table 2.7). Genotypic differences were found in biomass, grain size and HI. Tall plants had the greater biomass due to longer stem, and Semi-dwarfs were higher than Double dwarf. The

same trend was found for grain size and ranked as Tall > Semi-dwarf > Double dwarf, but the opposite was found for HI. At the whole plant level in Expt 3, genotypic differences were again observed for biomass, stem weight, grain size and HI but to a smaller extent. Double dwarf still showed the additive effect of semi-dwarfs in those traits except for grain size, where no difference was found between *Rht18* and Tall and Double dwarf. Differences for spike weight, grain weight and grain number among genotypes were not significant. This is the same as the results from the main stem (Table 2.7). No genotypic difference was found at plot level (Table 2.9), which may due to variation between plots. Further statistical comparisons between tall and semi-dwarf lines, *Rht18* and *Rht-D1b* and Double dwarf and *Rht-D1b* revealed no difference except for differences in HI between tall and semi-dwarf lines (Appendix Table 2.2). The difference in HI suggested the most prominent contrast is between tall and semi-dwarfs since the latter reduce plant heights to have higher HIs. Consistent with Expt 3, spike weight, spike number, grain yield and grain number were similar within genotypes.

Table 2.7 Summary data at maturity in Expt 1, Expt 2 and Expt 4 with significance test, the interaction between Genotype and Environment was not significant. Values are per main stem

Treatment	Biomass (g)	Spike weight (g)	Grain weight (g)	Grain No.	Grain size (mg)	Harvest index
Genotype						
Rht18	4	2.3	1.8	48.5	36.3	0.45
Rht-D1b	4.1	2.3	1.8	48.9	37.5	0.46
Tall	4.7	2.4	1.9	47.3	40.4	0.42
Double dwarf	3.5	2.1	1.6	48.4	33.8	0.47
l.s.d.	0.4***	ns	ns	ns	2.4***	0.04*
Environment						
Expt 1	-	2.1	1.7	47.5	35.1	-
Expt 2	3.5	2.1	1.6	40.2	39.8	0.46
Expt 4	4.6	2.7	2	56.8	36.1	0.44
l.s.d.	0.2***	0.2***	0.2***	4.3***	2.1***	ns

*: $P<0.05$, ***: $P<0.001$, ns: not significant

Table 2.8 Summary data at maturity in Expt 3 (Black Mountain). Values are averaged from a sample of five plants per line

Genotype	Biomass (g)	No. of tiller	Stem weight (g)	Spike weight (g)	Grain weight (g)	Grain No.	Grain size (mg)	Harvest index
Rht18	41.6	9.6	16.3	25.3	19.6	441	45	0.47
Rht-D1b	37.4	8.9	14.2	23.2	17.6	419	42	0.47
Tall	44.5	9.1	19.8	24.8	18.9	393	48	0.42
Double dwarf	34.4	8.7	12.6	21.8	16.7	380	44	0.49
l.s.d.	7.1*	ns	3.1**	ns	ns	ns	4*	0.03**

*: $P<0.05$, **: $P<0.01$, ns: not significant

Table 2.9 Summary data at maturity in Expt 2, values are per m^2

Genotype	No. of spike	Grain weight (g)	Grain No.	Grain size^ (mg)	Biomass (g)	Grain yield (g)	Harvest index
Rht18	363	405	10000	39.9	755	368	0.48
Rht-D1b	339	368	9100	40.5	854	404	0.47
Tall	327	355	8100	43.6	799	332	0.42
Double dwarf	342	376	10000	35.6	714	349	0.47

No. of spike, Grain weight, Grain No, Grain size and HI were based on quadrat (1.2 m × 0.3 m). ^Grain size was adopted from main tiller in the quadrat and Grain No. was calculated by Grain size and Grain weight. Grain yield and Biomass were based on plot (1.3 m × 6 m). Biomass was calculated by HI and Grain yield.

The HI was measured in two populations segregating for *Rht18* and *Rht-D1b/B1b* (Table 2.10). The results from both Espada and Young families showed that *Rht18* had a similar HI to *Rht-D1b/B1b*, and both were greater than the tall lines, which suggested that all three semi-dwarfing genes had potentially the same HI.

In summary, no difference was found for the examined traits in all levels between *Rht18* and *Rht-D1b*.

Table 2.10 Means of HI in Expt 5 and Expt 6 at GES 2014

Expt	Expt 5 (Espada)	Espt 6 (Young)	Averaged Genotype
Genotype			
Rht18	0.43	0.47	0.45
Rht-B1b	-	0.48	0.48
Rht-D1b	0.44	-	0.44
Tall	0.40	0.42	0.41
Double dwarf	0.47	0.48	0.47
Averaged Expt	0.44	0.47	

The l.s.d. was 0.03 (Genotype***), 0.02 (Expt **), and no significance was found at G×E. ***: $P<0.001$, **: $P<0.01$

2.4 Discussion

This chapter evaluates agronomic traits associated with *Rht18* with the prospect of it replacing the conventional dwarfing gene *Rht-B1b* or *Rht-D1b* in breeding programs. First it is important to assess data for plant height, internode length, flowering time and grain yield to determine whether *Rht18* behaves differently to *Rht-D1b,* which may compromise yield or adaptation. Also, it is interesting to see how the Double dwarf that contains *Rht18* and *Rht-D1b* behaves in order to understand the interaction of both genes in the same population.

Rht18 was found to reduce plant height by about 25% compared with the wild type Tall. This is the same as reported for *Rht18* in a Chinese cultivar Xifeng20, but higher than in another cultivar Fengchan3 (Yang et al. 2015). The height reduction by *Rht18* is equivalent to that found for *Rht-D1b* in several studies. Richards (1992a) had found that *Rht-B1b* or *Rht-D1b* reduced plant height approximately 23% in rainfed environments, which was larger than the 18% reduction of *Rht-D1b* under irrigated conditions (Fischer and Quail 1990). The stronger acting *Rht-B1c* was found to reduce height by approx. 47% under both rainfed and irrigated conditions in the above studies. The combination of *Rht18* and *Rht-D1b* reduced height by about 32% in this study, which is lower than 47% for the combination of *Rht-B1b* + *Rht-D1b* reported in rainfed environments (Richards 1992a) and also lower than the 43% for double dwarfs grown under irrigation according to Fischer and Quail (1990). It is notable that the combination of *Rht-D1b* and *Rht-B1b* is additive whereas the addition of *Rht18* to *Rht-D1b* is incremental. This suggests that the interaction between *Rht18* and *Rht-D1b/B1b* may be different from *Rht-B1b* + *Rht-D1b*. The Double dwarf combining *Rht-B1b* with *Rht-D1b* is too short in most instances and they have rarely been grown commercially. The small additional height reduction provided by *Rht18* in the presence of *Rht-D1b* may provide an important opportunity to adjust the plant height of lines with *Rht-B1b* or *Rht-D1b* without further compromising coleoptile length or seedling vigour in high yielding environments (see Chapter 3).

The individual internode lengths revealed the partitioning of stem length and percentages of each internode to the stem shows the pattern in reduced length affected by different semi-dwarfing genes. The proportional length of the different internodes was essentially the same for all genotypes in this study, although some of the small differences were significant; for example, both *Rht18* and *Rht-D1b/B1b* reduced the peduncle and P-1 proportionally but P-2 to a lesser degree compared with the Tall. The reduction in internode lengths attributed to *Rht18* in this study was different in magnitude (larger in this study) compared with that reported by Yang et al. (2015) but the trend was similar.

Across all the experiments, presence of *Rht-D1b* or *Rht18* did not alter time to anthesis. Richards (1992a) found very small differences in flowering time between different *Rht* genotypes. In his studies, tall lines flowered earlier than *Rht-B1b* or *Rht-D1b* by about 1.5 days and *Rht-B1b* or *Rht-D1b* was one day earlier than Double dwarf (*Rht-B1b* + *Rht-D1b*). However *Rht-D1b* was not found to be later in any experiment, which is consistent with Fischer and Stockman (1986). Therefore, flowering time difference of *Rht18* is minor and probably insignificant.

No differences between genotypes were found for grain yield when measured on a main stem, plant or plot basis. This contrasts with *Rht-D1b/B1b* being the driver of the 'Green Revolution' and conferring significantly higher grain yields than tall wheats (Fischer and Wall 1976; Jain and Kulshrestha 1976; Flintham et al. 1997). Studies that demonstrate the yield advantage of semi-dwarf wheats come from field plots. This was not always possible in this study as firstly, lines were being progressively developed and seed was often only available for single rows and not for plots, and secondly, severe bird damage destroyed the main field plot study (Expt 4) in the final study year. In the studies on single rows, it is not surprising that the Tall genotypes had larger biomass and the double dwarfs had the smallest biomass. When planted in rows the extra height can result in more light capture and hence more biomass. This extra light capture may also increase spike weight and grain number and

that is also evident in Yang et al. (2015), which led to the slight reduction in grain yield as well as the non-significant increase in HI in two backgrounds. It was encouraging that the ranking for HI and grain size on single plants is highly conserved, and are closely related to yield in plots (Quail et al. 1989). Accordingly HI was ranked inversely to plant height and grain size was ranked positively with plant height. It is also notable that these differences among genotypes were also highly significant. Furthermore, it is also encouraging that in the only plot trial that could be harvested (Expt 2), the semi-dwarfs tended to have more grain yields and grain number per unit area (although not significantly) than the Tall genotypes. It is also notable, and to be expected, that biomass differences between semi-dwarfs and talls were minor in the plot study. Thus, although there was no significant yield increase associated with *Rht18* and *Rht-D1b* no major disadvantage was found and in the only plot experiment that was harvested there was a trend for improved yields associated with the semi dwarfs.

An increased grain number, which typically explains the increase in yield associated with dwarfing genes was evident in the plot study but not when main stems or plants were sampled. This is not unexpected as grain number of single culms/plants do not generally translate to whole plots (Quail et al. 1989). In the combination of Expt 1, 2 and 4, *Rht18* and *Rht-D1b* tended to have more grains than Tall, although the difference was not significant. Also, Gale (1979) suggested that the success of *Rht-D1b* was due to more fertile tillers than in Tall genotypes. At the plant level, there was neither difference in number of tillers per plant nor in grain number between genotypes. However, Yang et al. (2015) showed *Rht18* increased grain number in both cultivars by producing more grains per spike rather than by more tillers per plant. According to Flintham et al. (1997) and Fischer and Quail (1990), Double dwarf containing *Rht-D1b* and *Rht-B1b* may further increase grain number. No additive effect of the *Rht18* and *Rht-D1b/B1b* genes was showed in this study. A likely reason for not seeing an increase in grain number is perhaps due to growth conditions such as limitations on seed supply as discussed in grain yield section. Data available from Expt 1,3,5,6 are from rows and

these conditions favour the tallest lines due to higher light interception and the dwarf lines are disadvantaged the most. This is evident in Expt 1 (data not shown individually) and Expt 3 (Table 2.8) where the grain number per main tiller or per plant was not different across genotypes, and the Double dwarf has the least grain number. In Expt 2, the grain number in plots was calculated from grain size and grain weight and it showed no difference probably due to the diversity of lines in the bi-parental population or the possibility of uneven soil nutrition.

A negative pleiotropic effect on grain size associated with *Rht-D1b* has been noted before Gale (1979) and this was confirmed here where both *Rht-D1b* and *Rht18* had smaller grains. *Rht18* was also found to have reduced grain size in two Chinese cultivars (Yang et al. 2015). *Rht18* reduced grain size to a similar degree compared with *Rht-D1b* at both main stem and plant level. Double dwarf lines reduced the grain size even further suggesting the possibility of additive effects. In field plots this is typically balanced by an increased grain number associated with dwarfing genes.

Plant height, internode length and grain yield were different across experiments, which was altered through the interaction with genotype. Plant height is highly variable at different growing conditions, in this case, sowing time (Expt 1 versus Expt 3), row spacing (Expt 4 versus Expt 5), and genetic background (Expt 5 versus Expt 6) were important in different experiments. G × E effect was most prominent in height and internodes reduction (Table 2.3, Table 2.4 and Table 2.5). For example in Expt 1 and Expt 4, tall lines were similar in height in both experiments, whereas there was a 10 cm difference for both *Rht18* and *Rht-D1b*. G × E effect was not so much as a ratio of internode to stem in percentage (Table 2.4), which suggests Tall and dwarfing genes will not change their proportions of internode length under different environments (Appendix Figure 2.1). No G × E effect was found on grain yield traits (Table 2.7, Table 2.9) indicating that different genotypes may respond similarly under different environments.

2.5 Conclusion

This study evaluated height, time to flowering, and grain yield and its components of *Rht18* in bread wheat compared with *Rht-D1b/B1b* in different populations to provide important data for future application of *Rht18* in breeding. *Rht18* had similar final plant height to *Rht-D1b* or *Rht-B1b*, which was 65-80% of Tall whereas the Double dwarf reduced the height to 60-70% of the Tall isoline. The distal three internodes were reduced in length in both semi-dwarfs and double dwarf lines, while there were no differences between *Rht18* and *Rht-D1b* or *Rht-B1b*. Length reduction was spread along all internodes of the culm of both *Rht18* and *Rht-D1b/B1b* rather than concentrated in a particular one.

The flowering time appeared to be very close among the tall and dwarf lines, which suggest both *Rht18* and *Rht-D1b* do not alter the timing of developmental events such as anthesis.

No genotypic differences were found for grain weight per spike or grain number. Grain size ranked as Double dwarf < *Rht18* = *Rht-D1b* < Tall. The grain yield of lines with the dwarfing genes could not be discriminated from Tall. Differences of stem weight or HI were due to reduced stem length, and single or double semi-dwarfs had significantly higher HI than Tall. There were no differences found in any of those traits between *Rht18* and *Rht-D1b/B1b*, thus there was no evidence of any yield penalty associated with *Rht18* and no evidence for it being different agronomically to *Rht-D1b/B1b*. This accords well for use of *Rht18* in breeding. But yield advantage for *Rht18* compared with Tall should be confirmed in larger field plots.

Chapter 3 Does *Rht18* affect coleoptile length, early leaf area or seed dormancy?

3.1 Introduction

This chapter examines whether *Rht18* affects seed and seedling characteristics such as coleoptile length, seedling emergence, early leaf area and seed dormancy by comparing lines containing *Rht18* with lines carrying *Rht-D1b* or *Rht-B1b* in hexaploid or tetraploid wheat populations.

Plant density and stand establishment after sowing are two key characteristics linked to yield. Seedlings that fail to emerge result in low plant density and emerged weak seedlings remain small. Crops with poor establishment are more vulnerable to environmental stress, are poor competitors with weeds, intercept less light and are likely to produce less yield. Thus, it is vital to achieve a high ratio of emerged plants with good establishment. Timely sowing is also crucial (Photiades and Hadjichristodoulou 1984), so that flowering and grain filling occurs at the optimum time. Early sowing can hasten development that increases the risk of frost damage (Single 1961; Nuttall et al. 2012), while late sowing can result in poor crop establishment and low yield (Kohn and Storrier 1970; Kerr et al. 1992). Delayed sowing has been associated with a reduction in biomass and grain yield in most of the Australian wheat belt (Doyle and Marcellos 1974; Shackley and Anderson 1995).

Most Australian wheat is produced under rainfed or water-limited conditions where up to 50% of the rainfall can be lost due to evaporation from the soil surface (Leuning et al. 1994). Sufficient soil water supply is essential to maximize both biomass and grain yield, and yield potential is often constrained by lack of water (Fischer 1979; Richards 1991). During the optimum sowing time, soil moisture is often scarce in these regions. To avoid delay in sowing, three main requirements must be met to have good crop establishment.

First, rapid growth of coleoptile enables quick emergence. When a wheat seed germinates, the radicle will first emerge together with seminal roots. The coleoptile, a sheath which surrounds the emerging leaves, follows shortly after, and increases in length until it reaches the soil surface (Figure 3.1). Fast emergence is desirable for earlier above-ground vegetative growth and improved water-use efficiency (dry matter produced per unit of transpired water). Coleoptile emergence can be described by the emergence rate index (ERI) which is calculated from three successive emergence counts (C), i.e., ERI=C1×3+C2×2+C1×1 (Allan 1980).

Second, long coleoptile length enables deeper sowing. In some years, insufficient moisture may be present in the surface soil for germination to occur at the optimum sowing time. If moisture is present deeper in the soil profile, deep sowing is an option allowing farmers to sow closer to optimum time (Mahdi et al. 1998; Schillinger et al. 1998). However, deep sowing often results in a low rate of seedling emergence and poor establishment because the coleoptile fails to reach the soil surface (Allan et al. 1962). Wheat cultivars with a short coleoptile have poor establishment when seeds are sown deeper than 5 cm. Longer coleoptiles are associated with greater early vigour, and provide a higher seedling ERI and greater field emergence when seeds are sown deeper than 5 cm (Allan et al. 1962; Sunderman 1964).

Third, rapid early leaf growth and larger leaf area of seedlings reduces loss of soil moisture through evaporation. Crops with greater seedling vigour are expected to achieve canopy closure faster and shade the soil surface to reduce evaporation and increase water availability for crop use. Faster emergence also enables plants to start photosynthesis earlier. Increased water-use efficiency leads to a larger plant biomass (Condon et al. 2002). According to López-Castañeda et al. (1996) and Rebetzke and Richards (1999) leaf width is highly correlated with early seedling vigour and can be used as a selection tool for breeders.

Coleoptile length and early leaf area can be affected by environmental and genetic factors, and this study focused on the latter. *Rht-B1b* and *Rht-D1b* are the most common semi-

dwarfing genes deployed in modern wheat cultivars, but they are associated with shorter coleoptiles (Fick and Qualset 1976; Allan 1989) and a reduction in leaf area (Richards 1992b). Not surprisingly, the number of seedlings emerged from a depth of 11 cm was found to be significantly lower in wheat lines carrying *Rht-B1b* or *Rht-D1b* compared with tall lines (Addisu et al. 2009). Richards (1992b) found that leaf area of leaf 1 and 2 was reduced by 7% in *Rht-B1b* or *Rht-D1b* compared with Tall near-isogenic lines. Rebetzke and Richards (1999) tested Australian and CIMMYT-derived semi-dwarf wheats containing *Rht-B1b* or *Rht-D1b* and found that both of these semi-dwarfing genes reduced seedling leaf area significantly by decreasing both leaf length and width. Thus, breeding for long coleoptile or greater seedling vigour with short statured wheat is limited by the presence of *Rht-B1b* or *Rht-D1b* in most wheat cultivars (Rebetzke et al. 2001).

Alternative height reducing genes were described by Konzak (1988), for example *Rht8, Rht12* and *Rht13*. These genes were identified as induced or spontaneous mutations that reduce plant height by up to 50% without affecting the coleoptile length (Rebetzke and Richards 1999; Ellis et al. 2004) indicating that variation in coleoptile length is independent of plant height. These GA responsive genes have the potential to replace the GA insensitive *Rht-B1b* or *Rht-D1b* in water-limited environments like Australia, and some have already been introduced into Australian germplasm (Rebetzke and Richards 2000b; Bonnett et al. 2001), although so far there are no commercial releases.

Some GA-insensitive dwarfing genes such as *Rht-B1c* have been linked with an important seed quality trait, resistance to pre-harvest sprouting (PHS). PHS occurs when physiologically mature grain germinates in the spike following rainfall and before harvest takes place. It results in downgrading grain from food quality to feed grain as physical properties of the dough deteriorate (Derera 1982). PHS is found in different regions of the world and causes significant damage to the harvested grain (Derera 1980). As an example, farmers from the Newcastle freight zone in New South Wales were reported to lose $M32.25

from 2.29 M tonnes of wheat due to early sprouting during 1977-1980 (Derera 1982). In 2003-2004 farmers in southern regions of the Australian wheat belt lost 20% of the value of their grain due to the same reason (Australian-Wheat-Board 2003–2004).

PHS resistance is improved by seed dormancy (Piech et al. 1970) which is traditionally associated with red grain colour in wheat breeding programmes (Gfeller and Svejda 1960). Australian wheat has a reputation for its white seed coat grains, and white grain wheats were associated with low dormancy. However, PHS resistance can be increased by integrating GA insensitive dwarfing genes such as *Rht-B1c*. *Rht-B1c* was found to inhibit the response of wheat aleurone to GA and it had been employed in a white grain wheat 'Tordo' to combat PHS (Bhatt et al. 1977). The widely known GA-sensitive semi-dwarf genes *Rht-B1b* and *Rht-D1b* had inconsistent PHS resistance and GA responsive genes were less studied and showed no promising result (Gooding et al. 2012).

Rht18 was identified as one of the most promising alternative semi-dwarfing genes showing good agronomic characteristics such as optimal height, long coleoptile, strong straw and grain fertility. It was first released as Icaro from a durum cultivar Anhinga in Italy in 1987. In a previous study, coleoptiles of Icaro carrying *Rht18* were shorter (18%) than the wild type Anhinga, suggesting that *Rht18* may reduce coleoptile length (Ellis et al. 2004). Coleoptile length and early leaf area development have not been evaluated in bread wheat populations containing *Rht18*, nor has any effect on seed dormancy been reported. In this study, we examined the effect of *Rht18* on coleoptile length and early leaf area and seed dormancy using populations segregating for *Rht18* and *Rht-D1b/B1b* developed in bread and durum wheat.

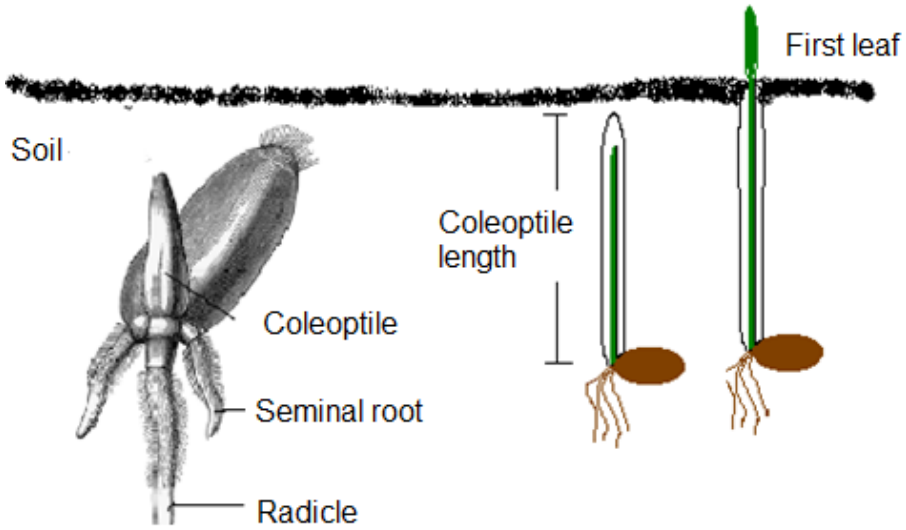


Figure 3.1 Following germination of the seed, the coleoptile protects the first leaf until it reaches the soil surface

3.2 Materials and Methods

3.2.1 Plant material

The study on early growth was conducted in three populations developed to characterize *Rht18* in bread and durum wheat (Table 3.1).

A bread wheat population was developed from a cross between Espada (*Rht-D1b*), and HI25M (*Rht18*) (material and methods Chapter 2). Approx. 300 F_2 seedlings were screened with the perfect marker for *Rht-D1b* (Ellis et al. 2002) and the SSR marker (*WMS4603*) that is tightly linked to *Rht18* (Spielmeyer et al. unpublished). Between 12 and 14 homozygous lines were identified for each genotypic category including *Rht18, Rht-D1b,* Tall and *Rht18+Rht-D1b* (Double Dwarf). This bi-parental population was advanced to F_3 (Expt 8) through single seed decent (SSD) to score coleoptile length and to F_7 generation (Expt 9) in the deep sowing trial in Ginninderra Experimental Station (GES), Canberra, ACT. Details of the GES site are provided in Materials and Methods in Chapter 2.

Another two bread wheat populations were developed by crossing the *Rht18* donor line HI25M with cultivars Espada and Young (*Rht-B1b*) and then backcrossed twice to each cultivar to generate BC_2F_2 populations segregating for *Rht18* and *Rht-D1b* in Espada, or *Rht18* and *Rht-B1b* in Young. Around 300 BC_2F_2 seedlings were screened with the *Rht18* and *Rht-D1b* or *Rht-B1b* markers to select 8-12 homozygous *Rht18*, *Rht-D1b* or *Rht-B1b*, tall, and double dwarf lines in both populations. BC_2F_2 plants were advanced to BC_2F_4 (Expt 10 or Expt 11 Table 3.1) in a glasshouse for early leaf area assessment and deep sowing experiments in trays. BC_2F_6 (Expt 7) seeds were harvested from heads from three lines per genotype of Expt 4 (Chapter 2) when spikes lost all green colouration (physiological maturity) for assessment of grain dormancy. Spikes were put in a fume hood for further drying for 48 hrs and then hand threshed. All populations consisted of four genotypes (*Rht18*, *Rht-D1b* or *Rht-B1b*, Tall and Double dwarf) and each genotype was represented by five independent lines (unless otherwise specified).

A durum population was generated by crossing homozygous short and tall progeny derived from a cross between Icaro (*Rht18*) and tall Langdon. Approx. 200 F_2 plants were screened with flanking SSR markers *barc3* and *gwm356* to identify 39 recombinants. The 39 plants were advanced to F_4 or F_5 generation (Expt 12) by SSD, and consisted of 24 short and 15 tall lines. A subset of 14 short lines and 14 tall lines was chosen for assessment of coleoptile length.

Table 3.1 Populations deployed in early vigour study with sowing dates. Abbreviations: Pop, population; Bkg, background; Dorm, dormancy; Col Asse, coleoptile assessment.

ID	Pop	Bkg	Parent 1	Parent 2 (recurrent)	Dorm test	Col Asse	Deep sowing	Early leaf area
Expt 7	BC_2F_6	Bread wheat	HI25M	Espada	6th Dec 2014			
Expt 8	F_3	Bread wheat	HI25M	Espada		April 2012		
Expt 9	F_7	Bread wheat	HI25M	Espada			14th July 2014	
Expt 10	BC_2F_4	Bread wheat	HI25M	Espada			3rd Oct 2014	25th July 2014
Expt 11	BC_2F_4	Bread wheat	HI25M	Young				25th July 2014
Expt 12	F_4/F_5	Durum wheat	Icaro	Langdon		April 2012		

3.2.2 Assessment of coleoptile length in bread and durum wheat

Around 11-14 lines per genotypic class with six seeds per line from Expt 8 and Expt 12 were sown in wooden trays (55 × 28 × 12 cm) containing a potting mix of 50% compost and 50% vermiculite. Trays were wrapped in black plastic to prevent moisture loss and to block out light and placed at 4 °C for 2 days to remove any residual seed dormancy and ensure even germination. Trays were then kept in a cabinet maintained at a constant 15°C for 14 days. Coleoptile length was measured as the distance from soil surface to the top of the coleoptile sheath (Rebetzke et al. 1999). Six coleoptile length measurements from each line were ranked and the three longest values (free from any abnormalities) were used to calculate the mean. Data was analysed by Genstat (V16th Edition) for ANOVA for main effects of genotype.

3.2.3 Effect of sowing depth on seedling emergence

The effect of sowing depth on emergence was assessed in controlled conditions in wooden trays (described in section 3.2.2) and in the field environment at GES. The first deep sowing

experiment was sown into trays using population Expt 10 (Table 3.1) on 3rd October 2014 under ambient conditions in Black Mountain, Canberra. Seeds with similar size were selected from four lines per genotypic class and sown in deep wooden trays with inner dimension 55 × 28 × 16 cm. Treatments included depth (9 and 12 cm), genotypic classes (*Rht18, Rht-D1b,* Tall and Double dwarf), with three replications. A soil layer was put at the bottom of the tray with a depth of 4 cm allowing root growth. Seeds were then laid on this layer at 10 per row, 16 rows per tray (rows were randomised), and additional soil was put onto the seed bed and levelled but not compressed. Trays were put outside and the soil supplied with sufficient water for seed germination. Emerged seedlings were scored two days after the first shoot had emerged for each treatment. Emergence of seeds of the same lines was also determined at 3 cm sowing depth with the same procedure in two trays (replicates). The scoring was destructive as each emerged seedling was traced back to its seed for correct alignment, which is why the Emergence Rate Index (ERI) test was not performed in this experiment.

The second deep sowing experiment was sown at GES using population Expt 9 (Table 3.1) on 14th July 2014. Four genotypes, each containing 4-5 lines with two replications were randomised into 40 plots (2.5 m × 1.5 m) and sown at 5 cm and 12 cm depth with 15 cm row spacing. About 20 gram seeds (approx. 500 grains) per line were sown in each plot. Stand establishment was calculated by counting the emerged seedlings along both sides of a one-metre ruler in each plot for 5 cm depth sowing, and counting the number of seedlings emerged from each plot for 12 cm depth. Both scores were converted to the number of plants per m^2. Two-way ANOVA analysis was performed by Genstat (V16th Edition) for variance among genotypes and treatments.

3.2.4 Assessment of seedling vigour

Assessment of early vigour was conducted in late July 2014 using population Expt 10 and Expt 11 from both Espada and Young background (Table 3.1). Each backcross had four genotypes with five lines per genotype and 4-5 seeds (replications) per line. All seeds were randomized across two trays. The seeds were selected to have a weight between 36 and 45 mg. Cultural details were as described in section 3.2.2. Plants were harvested when all seedlings had three fully expanded leaves on the main stem and the number of leaves and tillers (including coleoptile tiller) were recorded. Leaf width and length were measured with a ruler or calliper and leaf area was calculated using the formula 'Leaf area=0.75 × Leaf length × Leaf width'(Rebetzke and Richards 1999). Each leaf was dried at 60°C for 24 hours then weighed. Specific Leaf Area (SLA) was calculated as the ratio of leaf area to dry mass of the three main stem leaves. Total leaf area was calculated from the product of dry mass of the total leaves and SLA. Analysis of variance and means was performed by Genstat (V16th Edition) between genotypes with and without seed size as a covariant. Regression analysis between leaf width and total leaf area or biomass was performed by Sigmaplot (Version 12.3).

3.2.5 Seed dormancy

Grain germination tests were performed in plastic trays lined with moist Whatman 3MM paper to determine germination percentage (GP) and germination index (GI). After threshing, the seeds were placed (embryo down) on the trays and time was recorded as day 1 in T_0 (zero week of grain after-ripening). Four genotypes (12 lines) were tested with 100 seeds per line gridded on a 10×10 array on the filter paper. Trays were put into a growth chamber with continuous low intensity light (5-8 watt lamp) and a constant temperature of 20 °C. Paper was kept moist. The test was conducted during consecutive weeks with 7 days as a cycle. Observations were made every day (starting from day 2) and germinated seeds (radical

emergence) were removed and numbers were recorded. GP was calculated from the number of seeds germinated per line after 7 days and GI was determined via the formula $GI=1/2\times M_2+1/4\times M_4+1/6\times M_6$ (M_i means the number of seeds germinated between day $_{i-2}$ and day $_i$). The test was continued each week for lines with a germination percentage below 95%, and the test was terminated in T_2 when all the lines reached 95%. Germination percentage and index results for each genotype were analysed for means and ANOVA using Genstat (V16th Edition).

3.3 Results

3.3.1 Coleoptile length in bread and durum wheat

The coleoptile lengths of lines carrying *Rht18* were measured and compared with other lines without *Rht18* in both bread wheat and durum wheat to determine if *Rht18* has any effect on coleoptile length.

Coleoptile length of the bread wheat cultivar Espada (95 mm) carrying *Rht-D1b* was 16% shorter than the *Rht18* donor line HI25M (114 mm, Figure 3.2). The mean coleoptile lengths of lines carrying *Rht-D1b* or in combination with *Rht18* (Double dwarf) were similar to (or slightly shorter than) the Espada parent and 24-27% shorter than the *Rht18* and the Tall progeny lines. However, coleoptile length of lines that were only carrying *Rht18* were not different to tall lines, indicating that *Rht18* causes no reduction on coleoptile length in this bread wheat population.

In durum wheat, the average coleoptile length of the population was over 50 mm longer than that of the bread wheat population (Figure 3.2). There was a 20 mm difference between the two parents, but no difference between short and tall $F_{4/5}$ lines. The means of both progeny were midway between values from the parents. The result is consistent with *Rht18* having no effect on coleoptile length in bread wheat (see Discussion).

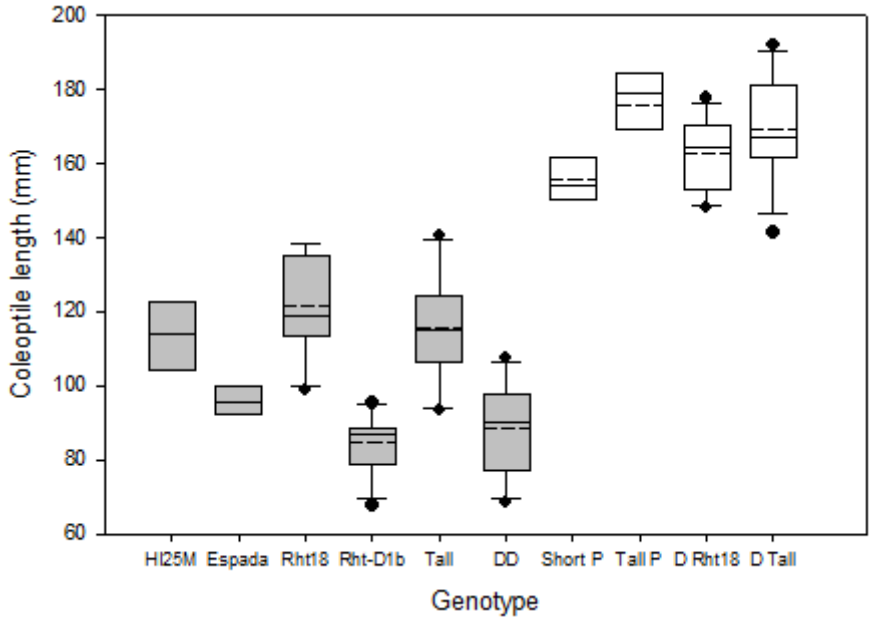


Figure 3.2 Coleoptile lengths of bread and durum wheat genotypes.
The grey boxes are bread wheat, and white boxes are durum wheat. Abbreviations: DD, double dwarf in bread wheat; Short P, Tall P, D *Rht18* and D Tall are short and tall parents, *Rht18* and Tall lines in durum wheat. The lower and upper edges of the box represent 25th and 75th percentiles, and the solid and dashed lines are the medians and means in each box. The 'error bars' indicates 10th and 90th percentiles; while the filled circles are mean outliers in each class.

3.3.2 Plant emergence following deep sowing

Emergence from deep planting is one of the key seedling vigour traits and it is believed to have a positive correlation with coleoptile length. The objective of the deep sowing experiments conducted in trays and in the field was to examine if the longer coleoptile *Rht18* has improved emergence than *Rht-D1b*.

In the tray experiment conducted at Black Mountain (climate data referred to material and method Chapter 2), deep sowing resulted in a significant reduction in seedling emergence. The emergence percentage from shallow sowing (3 cm) in trays (black bars in Figure 3.3) showed approximately 90% for all genotypes, and there was no genotypic differences

indicating an equal high germination percentage. The interaction between genotype and treatment was significant (Appendix Table 3.1). At a depth of 9 cm (grey bars), a greater number of seedlings emerged for *Rht18* and Tall compared with *Rht-D1b* and Double dwarf. The number of emerged *Rht18* lines was not different from tall lines suggesting that *Rht18* does not affect the percentage of emerged seedlings at this depth. Also, no differences were found between *Rht-D1b* and the Double dwarf. When seeds were sown deeper at 12 cm, the emergence percentage for all genotypes was reduced to approx. 10% and there were no genotypic differences**.** *Rht18* had a similar emergence as the Tall at all depths, and the Double dwarf (*Rht18*+*Rht-D1b*) was indistinguishable from semi-dwarf *Rht-D1b*.

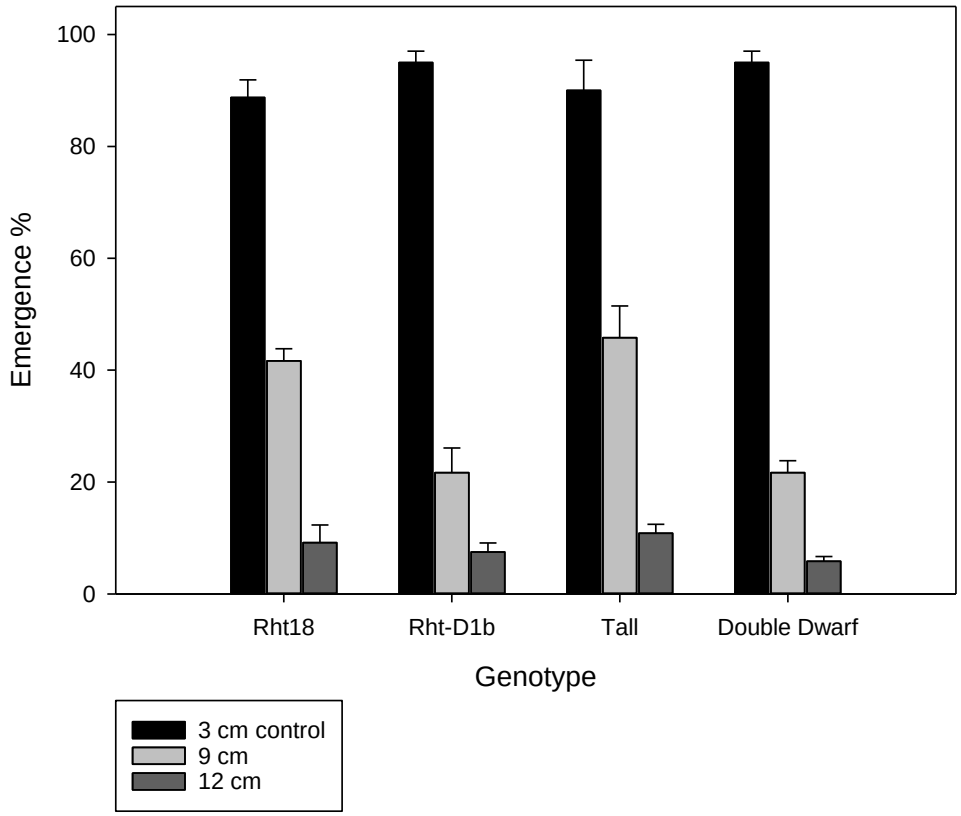


Figure 3.3 Emergence percentage of Expt 10 following deep sowing trial in trays. The interaction between sowing depth and genotype was significant at *P*=0.05.

Seeding at GES was compromised by uneven crusting of the soil surface due to rainfall after sowing, which increased the amount of variability in stand establishment, and the deep sowing treatment was the worst affected. The variance analysis in the GES plots showed that the emergence differences were derived from the sowing depths rather than from genotypes (Appendix Table 3.2). Deeper sowing reduced the emergence across both treatments as expected but no genotypic difference was found (Table 3.2). At 5 cm sowing depth, there were no genotypic differences, which suggested equal emergence between *Rht18* and *Rht-D1b* at shallow sowing. At 12 cm sowing depth, results in field plots were similar to those from trays. The emergence was so low that only 2-3% of the seeds emerged per plot, which was approx. 2 or 3 seedlings per m^2 and there were no genotypic differences. Nevertheless it is noteworthy that *Rht18* and tall lines had a higher emergence score (Figure 3.4). By combining data from the field and tray experiments (coleoptile length), the correlation between emergence and coleoptile length was positive at genotypic level with coefficients of determination r^2=0.93 (P<0.05) (Figure 3.4).

Table 3.2 Means of emergence at 5 cm and 12 cm depth from Expt 9 (the interaction between treatment and genotype is not significant)

Source	Mean (No. of plant per m^2)
Treatment	
5 cm	42
12 cm	3
l.s.d.	2***
Genotype	
Rht18	23
Rht-D1b	21
Tall	21
Double dwarf	23
l.s.d.	ns

***: P<0.001, ns: not significant

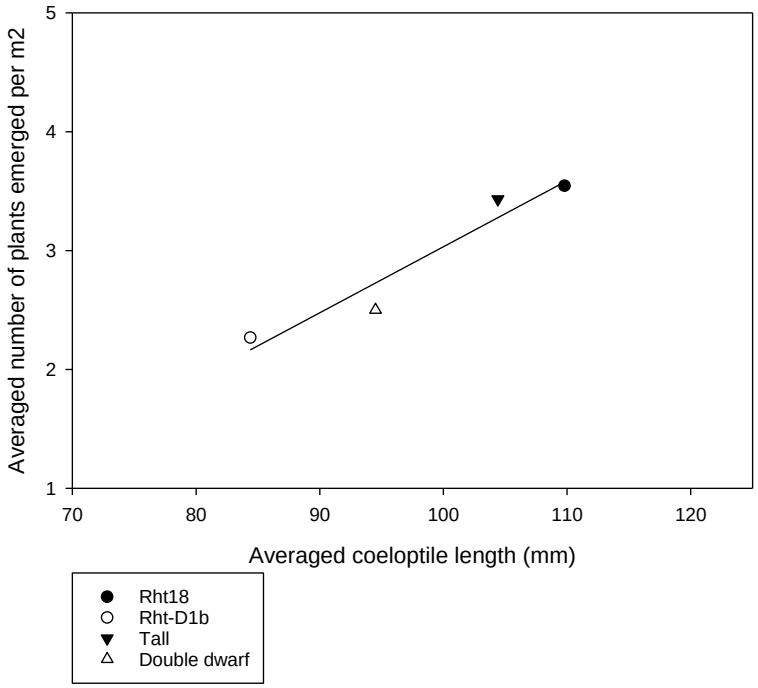


Figure 3.4 Averaged number of plants emerged per plot at 12 cm sowing depth (Expt 9) against mean coleoptile length (Expt 8) for each genotypic class ($P<0.05$)

3.3.3 Assessment of early seedling vigour

Traits associated with early vigour were determined in backcross derived lines in two genetic backgrounds to investigate the vigour of *Rht18* lines compared with *Rht-D1b* and Tall. Seed sizes were significantly different between the genotypes even though they were selected within a range (36-45mg). Seed size correlated with leaf width, total leaf area, total plant weight, leaf number and tiller number in Espada background, and with leaf width and length (Table 3.3) in Young background. To account for this association values in Table 3.3 are adjusted for variation in seed size.

Table 3.3 Means and least significant difference (l.s.d) adjusted by seed size as a covariate for early vigour components in Espada (Expt 10) and Young (Expt 11) backgrounds

Background	Geno-type	Seed size (mg)	Leaf No.	Tiller No.	LW mean (mm)	LL mean (mm)	SLA (cm^2/g)	Total PW (mg)	TLA (cm^2)
Espada	*Rht18*	42.6	3.4	1.8	6.01	87.2	289	95.7	16.5
	Rht-D1b	39.8	3.5	2.1	5.97	93.0	283	105	18.4
	Tall	42.1	3.5	1.9	6.15	93.8	292	109	19.5
	Double dwarf	39.8	3.4	1.9	5.89	85.2	279	97.3	16.7
	l.s.d.	1.5**	ns	ns	0.17*	3.6**	ns	10.3*	1.5**
	seed size		1.1*	1.5**	1.1**	ns	ns	1728*	39**
Young	*Rht18*	41.0	3.5	1.2	4.89	96.1	281	91.6	14.8
	Rht-B1b	38.8	3.5	1.0	4.94	98.6	284	86.3	14.9
	Tall	40.4	3.6	1.5	4.80	99.4	280	93.5	15.3
	Double dwarf	37.2	3.5	1.1	4.89	99.1	297	85.3	14.8
	l.s.d.	1.8**	ns	0.3**	ns	ns	ns	ns	ns
	seed size		ns	ns	0.43*	216*	ns	ns	ns

Significant level*, ** and ns indicates $P<0.05$, $P<0.01$ and not significant respectively. Seed size correlation was recorded in sum of the squares of the differences (SS) with significance levels. LW: leaf width, LL: leaf length, SLA: specific leaf area, PW: plant weight, TLA: total leaf area.

Genotypic differences in seedling vigour were found in the majority of traits in the Espada background but not in Young. In Espada, there were no differences in leaf number and tiller number indicating lines from all genotypes had the same development. *Rht-D1b* and Double dwarf had smaller averaged leaf width than Tall, while *Rht18* had no significant difference to Tall and *Rht-D1b* but closer to the latter after seed size correction. In terms of leaf length, *Rht18* and double dwarf lines were found to have shorter leaves than tall, while no difference was found between *Rht-D1b* and Tall. The specific leaf area (SLA) was uniform across all genotypic classes. For total plant weight and total leaf area (TLA), *Rht18* and

Double dwarf were smaller than *Rht-D1b* and Tall. *Rht-D1b* reduced both traits compared with Tall but not significantly.

In the Young background, all lines appeared to have longer but narrower leaves than lines in Espada. Genotypic differences were not found in any leaf area related traits, except that Tall had more tillers, which was not the case in Espada. Consistent with Espada background, *Rht18* had shorter averaged leaf length than *Rht-D1b* and Tall, although it was not significant. *Rht18* tended to have greater total plant weight than *Rht-D1b*, but equivalent total leaf area.

Across different genotypes, averaged leaf width was positively correlated with both total leaf area and total plant weight in two backgrounds (Figure 3.5), which confirmed that leaf width is a useful index to represent early vigour.

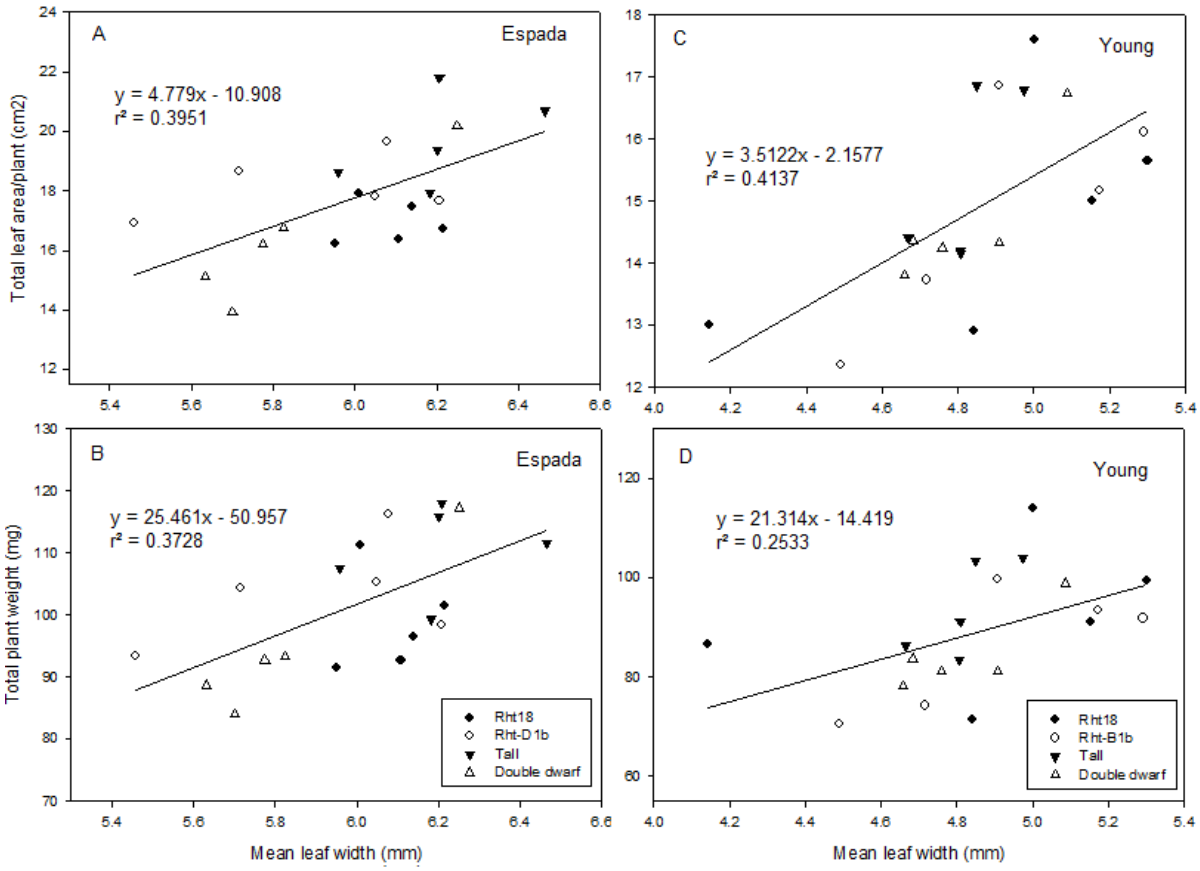


Figure 3.5 Relationship of mean leaf width (leaf 1, 2 and 3) with total leaf area and biomass per plant for 20 lines in Espada (A: r=0.63, *P*<0.01; B: r=0.61 *P*<0.01) and Young background (C: r=0.64 *P*<0.01; D: r=0.50, *P*<0.05)

3.3.4 Seed dormancy

Seed dormancy is an important index for grain quality and was examined in the Espada population varying for *Rht18* and *Rht-D1b*. Germination percentage (GP) and germination index (GI) were used to assess seed dormancy at the genotypic level.

There were no genotypic differences in averaged GP and GI as well as GP or GI in either of the weekly cycles (T_0 or T_1). There was a significant difference between T_0 and T_1 for both GP and GI indicating more grains germinated after one week of after-ripening (Table 3.4). At the end of T_1 which was 14 days after the initial germination test, all lines reached 95% GP. There were no significant differences identified between genotypes in this population, which indicates that *Rht18* and *Rht-D1b* have little effect on seed dormancy.

Table 3.4 Means of germination index and percentage for different genotype at T0 and T1

Genotype	T_0	T_1	Average genotype
#Germination percentage (%)			
Rht18	83.7	96.5	89.8
Rht-D1b	90.3	96.0	93.0
Tall	93.0	97.3	95.1
Double dwarf	92.3	97.7	94.9
Average T	90.1	96.9	
Germination index			
Rht18	18.7	32.7	25.4
Rht-D1b	24.3	37.0	30.4
Tall	24.1	37.3	30.4
Double dwarf	27.0	40.9	33.7
Average T	23.7	37.2	

Variation test showed differences for Genotypes and Genotype × T were not significant l.s.d.=7 for T in GP (*) and GI (***).

*: $P<0.05$, ***: $P<0.001$

3.4 Discussion

3.4.1 Coleoptile length in bread and durum wheat

This study has shown the effect of *Rht18* on coleoptile length within a durum population for the first time. Coleoptile length was longer in durum wheats than equivalent bread wheat lines, suggesting coleoptile length may be influenced by the ploidy level. A similar result was reported by Trethowan et al. (2001) where durum wheat generally has longer coleoptiles than bread wheat regardless of its height. A previous study concluded that Icaro (*Rht18)* had a shorter coleoptile than wild type Anhinga (Ellis et al. 2004). However, in this study the mean coleoptile length in short and tall durum progeny was the same. It is possible that results in Ellis et al. (2004) were confounded by background mutations that were induced by random mutagenesis and that may have reduced coleoptile length in Icaro, while these mutations were not expected to be present in wild type Anhinga. It is evident that the mean value of short and tall $F_{4/5}$ lines was midway between parental values, suggesting segregation of other genes in the population. Thus it is important to backcross the *Rht18* mutation into wild type or study segregating populations before the effect of *Rht18* on coleoptile length can be reliably assessed. In bread wheat, *Rht18* was recently reported to have no effect on coleoptile length compared with tall parents in two Chinese cultivar backgrounds (Yang et al. 2015). Results in this study confirmed that *Rht18* is unlikely to cause any reduction in coleoptile length in both durum and bread wheat.

High soil temperature can reduce the coleoptile length by up to 50% within the temperature range from 5-35 °C, and this experiment was conducted at the optimum temperature of 15 °C for wheat (Bhatt and Qualset 1976) allowing for a maximum coleoptile length. During the optimum sowing time, especially when soil temperature is high, other factors that limit coleoptile growth have to be considered such as adopting germplasm without *Rht-B1b* or *Rht-D1b*. According to this study, lines containing *Rht-D1b* were found to have

significantly shorter coleoptiles than tall lines, which was consistent with previous findings that *Rht-D1b* has strong negative effect (approximately 34% reduction) on coleoptile length (Ellis et al. 2004). Unfortunately, lines carrying *Rht-B1b* were not available in durum for this experiment, but *Rht-B1b* was found to reduce coleoptile length approximately 30% in durum wheat as in bread wheat (Trethowan et al. 2001). The alternative dwarfing gene *Rht18* appeared to have no negative effect on coleoptile length, similar to other GA-responsive genes such as *Rht8, Rht12* and *Rht13*, and this group of dwarfing genes have the potential to be sown deep.

3.4.2 Plant emergence following deep sowing

Plant emergence following deep sowing is affected by both the genetic background and environmental factors. Coleoptile length is a key driver to determine the deep seeding ability since it can explain 62-71% of the variability in emergence (Schillinger et al. 1998). Longer coleoptiles help to push through the soil and deliver the first leaf to the surface, while plants with shorter coleoptiles may end up exposing the first leaf below the soil surface, which then fails to emerge. Consistent with these previous studies, plants with long coleoptiles had greater emergence percentage in both the trays and field experiment at GES.

However an environmental factor, soil texture, plays an important role in determining whether the first leaf can emerge. The soil filled in the trays was not compacted or even crusty, so the germinated seeds could emerge even when the coleoptile was shorter than the depth of sowing. Under such conditions, it is not the coleoptile but the first leaf that pushes through the soil surface (Simmons 1987). Thus it is ideal to score the emergence ratio at an interval following the first leaf appearance, and the result should be informative to explain the deep sowing ability since the rate of coleoptile elongation is significantly correlated with emergence capability (Allan et al. 1961; Allan et al. 1965).

The emergence in the field is more difficult especially when soil crusting is present. Soil crusting or capping occurs worldwide under a range of weather conditions in many soil types. The impedance to the emerging seedlings from soil crusting is due to imposed mechanical resistance, with or without limitations from moisture, oxygen, soil temperature and planting depth (Awadhwal and Thierstein 1985). The seedling development force competes with resistance from crusting to determine the emergence of seedlings. If the development force falls short of the resistance force, the seedling will bend beneath the crust and fail to emerge. At GES, deep sowing was compounded by soil crusting after heavy rainfall resulting in very poor emergence (approx. 2-3%). While the ranking of coleoptile length between the trays and field experiments was similar, the validity of result should be improved by further experiments. For example, by sowing in fields with different soil textures: soft, medium and hard and across seasons to investigate the emergence ability of *Rht18* in the same population but in different environments.

Mohan et al. (2013) tested 662 wheat cultivars worldwide and confirmed coleoptile length was correlated with emergence percentage, but with a much lower coefficient (28%) compared with other studies, suggesting that seedling emergence from deep sowing could be driven by other factors. Coleoptile diameter as another genetic trait was associated with increased shoot strength and seedling emergence through crusted soil (Andrews et al. 1997). Coleoptile length and diameter were genetically independent (Rebetzke et al. 2004). The diverse germplasm adopted in Mohan's research plus traits like coleoptile diameter may contribute to lower variability of the relationship between emergence ratio and coleoptile length. Thus the effect on coleoptile diameter of the alternative dwarfing gene *Rht18* is certainly of interest to investigate in future.

3.4.3 Seedling vigour

Studies on early seedling growth did not reveal large differences between *Rht18* and other genotypes in the Espada and Young backgrounds, although in Espada, the *Rht18* lines were not as vigorous as *Rht-D1b* and the Tall genotype based on total plant weight and TLA. This contrasts somewhat with other studies where the Tall is more vigorous than *Rht-D1b* or *Rht-B1b* (Richards 1992b; Rebetzke et al. 2007). The most likely reason could be due to the genetic background where Espada as an elite cultivar favours high vigour performance of *Rht-D1b*. Further study for *Rht18* and *Rht-D1b/B1b* in other backgrounds will explain if the effect of *Rht-D1b* on leaf area or biomass can be found in other germplasm.

Seed size was shown to have a positive relationship with leaf area, dry weight, leaf length, leaf width, leaf number and tiller number and it accounted for 88-100% variation in seedling traits across cultivars (Richards and Lukacs 2002). In this study, seed size was shown to correlate with a number of traits in lines derived from Espada.

A coleoptile tiller is a second source of tillering that arises below the ground from the coleoptilar node. The coleoptile tiller has its own leaf area, and its growth was positively correlated with larger leaf area (Rebetzke and Richards 1999), and its presence varied with many genotypic and environmental factors (Liang and Richards 1994; Fujita et al. 2000). There was no difference observed across the genotypes for the presence of the coleoptile tiller in this research (data not shown), and its leaf area was incorporated into the total leaf area. Leaf width has been reported as a simple and effective trait to select for greater vigour as it incorporates embryo size and SLA (López-Castañeda et al. 1996). In both backgrounds, leaf width showed a positive relationship with total leaf area and plant biomass, which confirmed the importance of leaf width as an indicator for early vigour. However, the leaf width in the Young background was too narrow to identify any genotypic differences. Selecting an

appropriate genetic background is important to study leaf width, as genotypic variations could be easily shown at wider leaf width in Espada than in Young.

SLA showed no difference among genotypic classes, and no correlation with seed size. The mean values were very similar between Espada and Young, which suggested that SLA may not be influenced by the dwarfing genes.

3.4.4 Seed dormancy

The widely used GA-insensitive semi-dwarfing genes *Rht-B1b* and *Rht-D1b* had inconsistent PHS resistance (Gooding et al. 2012), and seed with *Rht-D1b* in this study did not express greater dormancy compared with the Tall. The GA-insensitive dwarf *Rht-B1c* had outstanding PHS resistance over *Rht-D1b* or *Rht-D1b* (Bhatt et al. 1977; Derera et al. 1977), and a better understanding of the underlying mechanism is required. This study compared the GA responsive gene *Rht18* with *Rht-D1b* and the double dwarf and tall lines in a near isogenic background. The result showed that both genes had no significant effect on dormancy, but different growth environments and repetition over years should be investigated to confirm the result.

3.5 Conclusions

The effect of *Rht18* on coleoptile length was studied in both bread and durum wheat. The results showed that *Rht18* had no effect on coleoptile length in both backgrounds, and indicated that *Rht18* could replace *Rht-D1b* in future cultivars to provide longer coleoptiles. The emergence percentage from deep sowing was positively correlated with coleoptile length at 12 cm depth in the field trial, which confirmed that *Rht18* had improved emergence than *Rht-D1b*. The emergence percentage from trays showed *Rht18* had the ability to establish

from deep sowing as Tall, and both performed better than *Rht-D1b* from 9 cm and 12 cm sowing depths although the data from 12 cm was not significant. The result suggested *Rht18* has potential to be used in future breeding programs.

There was some evidence that seedling vigour was slightly reduced in *Rht18* lines in the Espada background but not in the Young background. However, this effect was small. *Rht-D1b* reduced leaf width to have smaller leaf area and plant weight, while *Rht18* reduced more in length rather than width to have further decrease in leaf area and plant weight. The Double dwarf had no further reduction in seedling vigour compared with *Rht18* or *Rht-D1b/B1b*, suggesting there is no additive effect of the two dwarfing genes. This contrasts with the reduced vigour of conventional double dwarfs (*Rht-B1b* + *Rht-D1b*). The leaf width proposed to be used as a fast and non-destructive index in breeding program has been confirmed in this study to positively correlate with total leaf area and plant weight.

No evidence was found in this research that *Rht18* affects seed dormancy.

Chapter 4 Effect of *Rht18* on growth of the stem and spike, and mobilisation of apparent stem-stored dry matter to grain growth

4.1 Introduction

A critical period of growth in wheat that is associated with grain yield is from the start of stem elongation, i.e. terminal spikelet (TS) to anthesis, when spike and stem both grow very rapidly while relying on limited carbon supply (Kirby 1988). Wheat with *Rht* genes such as *Rht-B1b* and *Rht-D1b* are known to have more fertile florets at anthesis (Siddique et al. 1989), and greater grain number at maturity (Gale et al. 1985). The hypothesis was proposed that dwarfing genes like *Rht-B1b* and *Rht-D1b* reduce stem growth, resulting in less competition between spike and stem growth as a greater proportion of assimilates is partitioned to spikes than to stem during the critical period (Brooking and Kirby 1981). Similarly, Fischer and Stockman (1986) showed that heavier spikes or a greater proportion of spike weight to stem (or total) weight was observed in *Rht-B1b* and *Rht-D1b* lines compared with tall lines at anthesis. Later the competition hypothesis was supported by Gonzalez et al. (2011) based on the strong positive association between fertile florets and spike dry weight at anthesis.

Rht-B1b and *Rht-D1b* were reported to have no effect on the timing of developmental events such as initiation, duration, or termination but they may change the growth rate of stem elongation or dry matter relocation (Youssefian et al. 1992a). Elongation of internodes was initiated sequentially, and longer distal internodes had faster growth rates than basal ones. The maximum rate of stem elongation occurred just before anthesis when peduncle and penultimate internodes were elongating rapidly. *Rht-B1b, Rht-D1b, Rht-B1c* were found to slow down internode elongation rate, resulting in shorter and lighter internodes (Fischer and Stockman 1986; Youssefian et al. 1992b). However spike growth did not follow the same pattern as the internodes. The rachis elongation initiated after TS and reached full length

before ear emergence (EE), and linear spike growth in dry matter lagged behind. Fischer and Stockman (1986) found no difference in spike weight between semi-dwarf lines with *Rht-B1b* /*D1b* and Tall, but the proportion of spike to stem was larger in semi-dwarf lines in the 15 days preceding anthesis. Similarly, according to Youssefian et al. (1992b), there was no difference between *Rht-B1b*, *Rht-D1b*, *Rht-B1c* and tall lines in terms of spike length elongation, and lines with dwarfing genes had even faster growth rates than tall lines in dry matter accumulation, and this resulted in greater spike biomass throughout development till anthesis. In the same study, lines with dwarfing genes showed significantly greater ratios of spike against stem (dry matter) than tall lines, even before TS. Detailed studies for stem and spike growth for other dwarfing genes have not been reported so far.

During grain filling, there is a reduction in stem dry weight as a result of remobilisation of the stored water soluble carbohydrate (WSC). A portion of WSC is used as an important carbon resource for grain filling (Schnyder 1993). WSC can reach more than 40% of stem dry weight after anthesis (Blacklow et al. 1984) and up to 73% of this can be mobilised to the grain (Austin et al. 1980b). Stored WSC contributes more to grain weight under drought conditions than non-stressed conditions (Austin et al. 1977; Bidinger et al. 1977; Bell and Incoll 1990). The apparent contribution of stored WSC to grain yield in wheat is measured by changes in dry weight of stem, which depends on stem storage capacity and WSC relocation efficiency (Ehdaie and Shakiba 1996). Stem storage capacity correlates with stem length and specific weight (weight per unit length) or linear density (g cm^{-1}) (Blum et al. 1994). According to Ehdaie et al. (2006), more than 50% dry matter is stored in the basal internodes, the longer base internodes have more potential to store assimilate. On the other hand, the efficiency of stored WSC mobilised and translocated to grain is reported to be more related with loss of linear density of each internode than their length (Cruz-Aguado et al. 2000). Semi-dwarfs *Rht-B1b* exhibited greater contribution than tall lines in top two

internodes under drought conditions (Shakiba et al. 1996). Whether the semi-dwarfing genes *Rht-B1b* or *Rht-D1b* remobilise assimilates more efficiently than tall lines under non-irrigated conditions needs further study. Remobilisation of WSC during grain development for GA responsive dwarfing genes has only been reported for *Rht12*, where the capacity of dwarf lines to contribute dry matter to grain filling was reduced under irrigated conditions (Chen et al. 2013).

This chapter examines the impact of the semi-dwarfing *Rht18* gene compared with *Rht-D1b* on stem and spike growth (length and weight) in a set of closely related wheat lines grown at different sowing times and density. Also, changes of stem weight assumed to reflect changes of WSC were investigated after anthesis. Thus measurements were performed from TS to maturity but results are presented for pre and post anthesis. The objective was to measure differences of growth pattern for the standard (*Rht-D1b*) and new dwarfing gene *Rht18*.

4.2 Materials and methods

4.2.1 Plant material and cultivation

Three populations used in this chapter are listed in Table 2.1 Chapter 2. Expt 1, F_5 families were derived from the biparental cross HI25M (*Rht18*) × Espada and Expt 3, BC_2F_4 families were derived from the same cross. Both populations were sown in single rows spaced by 30 cm at Black Mountain. Expt 1 was sown in September 2012 while Expt 3 was sown in May 2013, which resulted in large difference between two experiments. Expt 1 and Expt 3 were harvested at 13 and 15 times for main stem in five plants from terminal spikelet (TS) to maturity respectively but spike and stem weight data were collected for 12 and 13 sampling times due to small weight measurements in each experiment. Expt 4 BC_2F_5 progenies of Expt 3 were sown in GES in plots (1.3 m × 6 m) in June 2014 and random five main stems were

only harvested at three times (10 days before anthesis, 7 days after anthesis and 28 days after anthesis). There were four genotypes in every experiment and there were five lines per genotype. Detailed cultivation and weather information can be referenced from Section 2.2.1 Chapter 2.

4.2.2 Genotyping

Genotyping details can be referenced from Section 2.2.2 Chapter 2.

4.2.3 Morphological measurements and data analysis

Stems were separated into component internodes (Figure 2.1 Chapter 2). Growth of internodes and spikes in length and weight was studied over time to determine if there was competition between stem and spike before anthesis. After anthesis, assuming WSC in internodes was remobilised to grain growth, changes in internode dry weight were measured to determine the contributions of each internode to grain growth. Internode and spike length measurements were undertaken as described in Section 2.2.3 Chapter 2. All internodes were cut at the node and the leaf sheath was removed. Spikes were cut at the base of the ear (node between ear and peduncle), and spike length was measured from the base to the tip of the ear in mm (not including awn). Spike length at terminal spikelet (TS) (39 DAS in Expt 1, and 85 DAS in Expt 3) was used in this chapter to examine the genotypic difference of apex stage. Spike weight, internode weight and linear density were determined after drying at 65 °C for 48 hours. Spike stem index (SSI) was calculated by dividing spike weight by the weight of spike plus stem (stem dry matter without leaf lamina dry matter and leaf sheath dry matter) at each sampling time. The linear density of each internode was calculated as the weight against the length and recorded as mg mm^{-1}. Change in WSC storage was calculated from the change

in dry weight for each internode. Fruiting efficiency (FE) calculated by grain number against spike dry matter at anthesis per spike for Expt 1, 3, and 4 respectively.

Statistical analysis was performed for the effect of genotype using ANOVA in Genstat (V16th Edition) and the least significant difference (l.s.d.) was provided. The length data were fitted using sigmoidal curves whereas weight data were presented by line and scatter plots. Thermal time (ºCd) was calculated by days multiplied by averaged daily temperatures for the period in question extracted from a temperature logger placed at each site starting from sowing day. Major growth period of spike (MGPS) in length and weight were determined using the corresponding thermal time to the middle 80% increase (between 10% and 90%) in length and weight of Expt 1 and Expt 3.

4.3 Results

Comparing lines with *Rht18* to lines with the reference gene *Rht-D1b* can help determine whether *Rht18* alters spike and stem competition by allocating more assimilates to the spike at anthesis and if remobilisation patterns differ between the genotypes.

4.3.1 Pre-anthesis growth of spike and internodes

4.3.1.1 Growth of spike and stem in length and weight

The increase in spike and total stem length and weight was plotted against thermal time for four genotypes in Expt 1 and Expt 3 (Figure 4.1 and Figure 4.2). Both Expt 1 and Expt 3 were derived from the same cross HI25M×Espada (Table 2.1 Chapter 2) allowing four genotypes to be compared in the same background in different environments. The advantage of Expt 3 was that it was backcrossed to Espada twice to have more uniform genetic background, and the earlier sowing for Expt 3 allowed maximum expression of stem length to show genotypic differences.

Spike length at terminal spikelet (TS) showed no genotypic differences in two populations (Table 4.1), suggesting both *Rht18* and *Rht-D1b* have equivalent spike developmental stage at the initial harvest. Also, no differences were found in spike length between different experiments suggesting lines from both experiments were at the similar growth stage.

Table 4.1 Averaged spike length at TS in Expt 1 and Expt 3. No significant difference was found at genotype, experiment or G×E.

Genotype	Spike length (mm)	
	Expt 1	Expt 3
Rht18	2.2	2.3
Rht-D1b	2.6	2.3
Tall	2.8	2.3
Double dwarf	2.5	2.5

For spike growth, there were no significant differences at each sampling time, but there were some differences when averaged over all sampling times (Appendix Table 4.1 and Table 4.2). There were differences in spike length in the biparental population but not in the backcross population. Tall and *Rht-D1b* had similar spike weight, and both were heavier than *Rht18* in Expt 3, but not in Expt 1 (Table 4.2). In Expt 4, spikes of *Rht18* had less dry matter than *Rht-D1b* at booting or one week after anthesis, while there was no effect on spike length. Differences in spike dry matter diminish at the later time point of 4 weeks after anthesis, suggesting it is possible that *Rht18* delay the growth of spike pre-anthesis (Table 4.3).

Table 4.2 Genotypic means across time for Expt 1 (biparental) and Expt 3 (backcrossed) populations from TS to anthesis (Abbreviation: *D1b*, *Rht-D1b*; DD, Double dwarf)

Geno type	Expt 1				Expt 3			
	Spike length (mm)	Spike weight (mg)	Stem length (mm)	Stem weight (mg)	Spike length (mm)	Spike weight (mg)	Stem length (mm)	Stem weight (mg)
Rht18	53	281	210	360	61	384	362	1126
D1b	52	299	236	378	61	424	357	1093
Tall	55	308	367	586	60	422	464	1436
DD	50	248	188	289	60	407	312	952
l.s.d.	3*	39*	19***	37***	ns	27*	10***	53***

*: $P<0.05$, ***: $P<0.001$, ns: not significant

Table 4.3 Averaged spike length, weight and harvest index and internodes length and weight per spike at three sampling times in Expt 4. [Abbreviation and units for SL, SW, PL, PW and PD: spike length (mm), spike weight (mg), peduncle length (mm), peduncle weight (mg)]

Genotype	SL	SW	PL	PW	P-1 L	P-1 W	P-2 L	P-2 W	SSI
Sampling 1: 10 days before anthesis (Booting)									
Rht18	94.2	232	27.2	18.4	47.4	66.4	85.9	206	0.29
Rht-D1b	95.6	319	41.4	29.9	64	93	82	217	0.34
Tall	93.4	337	52.8	39.6	90.7	141	109.8	287	0.28
DD	94.8	291	34.8	24.4	56.4	80.5	76.8	201	0.37
l.s.d.	ns	32**	6.4**	5.8**	9.3**	18**	5.8**	19**	0.02**
Sampling 2: 1 week after anthesis									
Rht18	96.4	712	261	309	150	378	101	386	0.34
Rht-D1b	95.3	774	282	353	164	426	92.2	369	0.34
Tall	93.4	758	376	468	213	579	120	450	0.28
DD	94.4	803	246	304	145	383	89.4	358	0.37
l.s.d.	ns	60*	17**	32**	7**	41**	8.7**	30**	0.02**
Sampling 3: 1 month after anthesis									
Rht18	88.9	2728	277	307	146	349	99.7	323	0.69
Rht-D1b	87.9	2771	286	320	160	427	87.9	295	0.69
Tall	82.2	2604	378	424	205	515	123.9	407	0.61
DD	86.2	2589	237	233	141	293	87.2	246	0.73
l.s.d.	2.7**	ns	12**	35**	7.7**	57**	9.3**	59**	0.02**

*: $P<0.05$, **: $P<0.01$, ns no significance

4.3.1.2 Major and maximum growth of spike and stem in length and weight

Identifying the relative timing for maximum spike and stem growth helps to understand when there is likely to be competition for carbon, and the mechanism of different dwarfing genes on stem elongation and how they interact with each other. MGPS is indicated by green and blue bars, where maximum growth was estimated at the middle of each bar (Figure 4.1 and Figure 4.2). MGPS for length and dry weight increase of the stem and spike were not the same. The MGPS coincided with early stem elongation in length but with mid-stem elongation in weight in both populations. The period of increase in dry weight for the spike overlapped with the dry weight increase of the stem. Therefore, competition must have occurred for assimilate between growth of stem and spike.

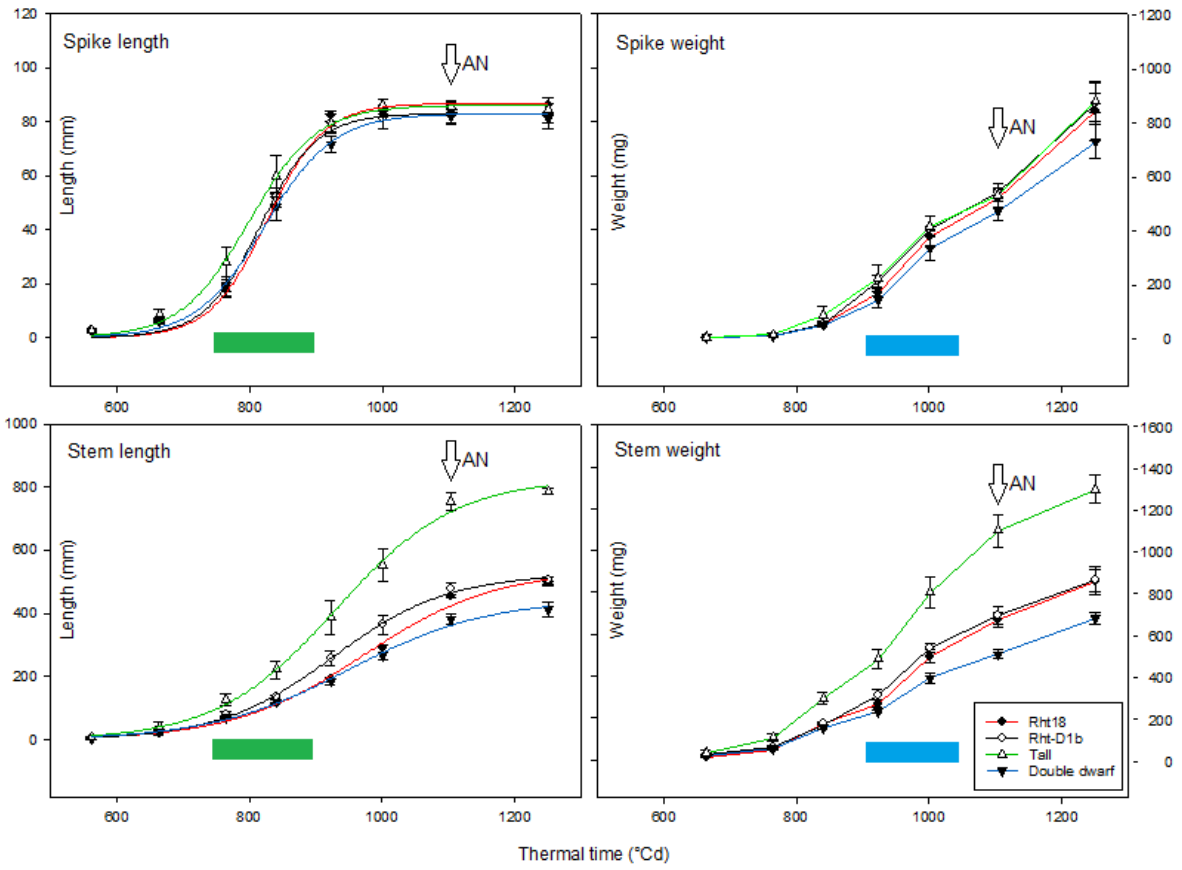


Figure 4.1 Length and weight changes over time for spike and total stem in Expt 1. Bars represent the standard error. AN means anthesis. Green and blue bars indicate Major Growth Period of Spike (MGPS) in length and weight respectively. Spike and stem elongation time dots were fitted in a 3-parameter sigmoid model in SigmaPlot (Ver. 12)

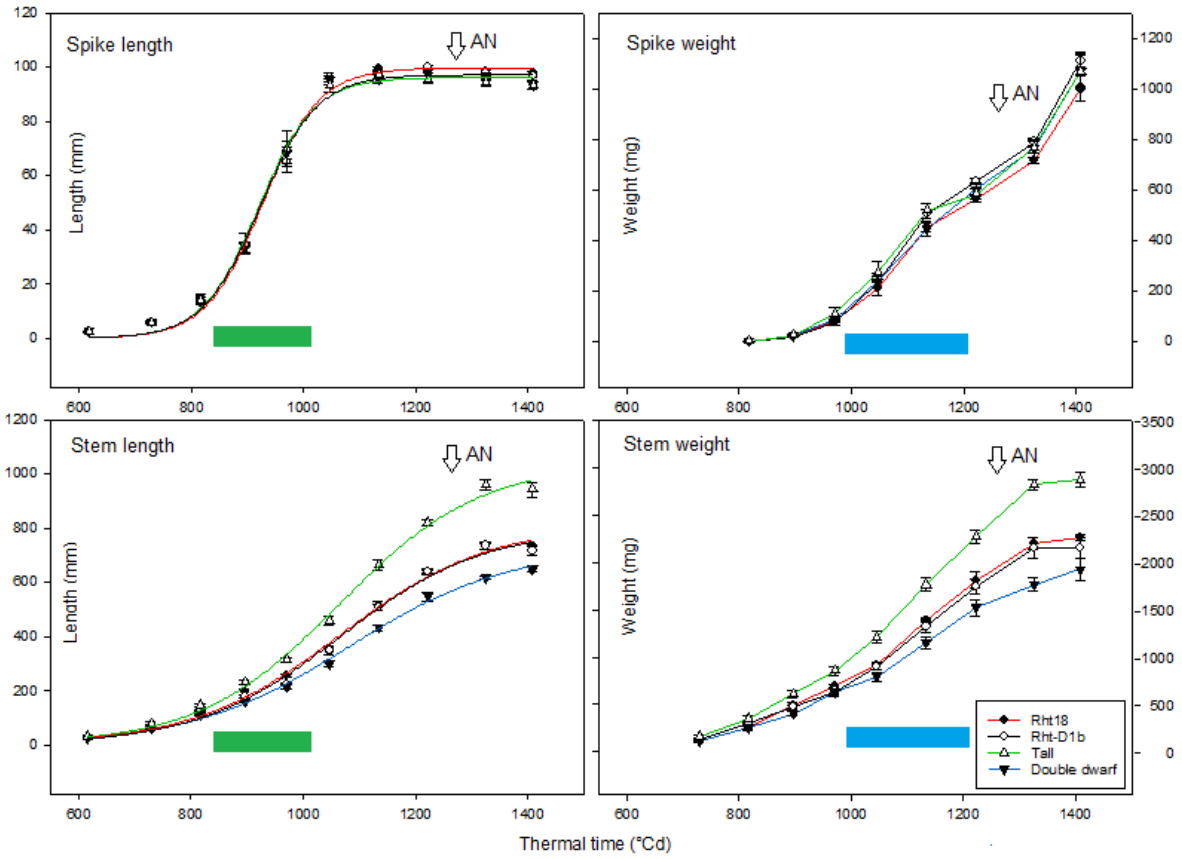


Figure 4.2 Length and weight changes over time for spike and total stem in Expt 3. Bars represent the standard error. AN means anthesis. Green and blue bars indicate MGPS in length and weight respectively. Spike and stem elongation time dots were fitted in a 3-parameter sigmoid model in SigmaPlot (Ver. 12)

4.3.1.3 Internode growth in length and weight

In order to study which internode competes with spike growth, and if *Rht18* differs from *Rht-D1b* in length elongation and weight accumulation during the critical period, stems were partitioned into peduncle, P-1, P-2 and P-3 (or including lower internodes). Detailed growth patterns for each internode were presented for Expt 1 and Expt 3 (Figure 4.3 and Figure 4.4) at genotypic level.

The MGPS occurred at almost the same time (Tt=750-900 in length and Tt=900-1050 in weight in Expt 1, Tt=850-1050 in length and Tt=1000-1200 in weight in Expt 3) across

four genotypes in both biparental (Expt 1) and backcross (Expt 3) populations (Figure 4.1 and Figure 4.2), indicating that *Rht18* and *Rht-D1b* do not differ in the timing of spike growth. It is interesting that maximum spike growth coincided with internode P-2 in length but with P-1 in weight in both populations (Figure 4.3 and Figure 4.4). It is also evident that the MGPS in weight overlapped primarily with dry weight growth of internode P-1 but also with the peduncle and with P-2 (Figure 4.4). Unlike the spike, which first increased in length and then in weight, resulting in a time interval gap for two sampling times, internodes increased length and weight at the same time.

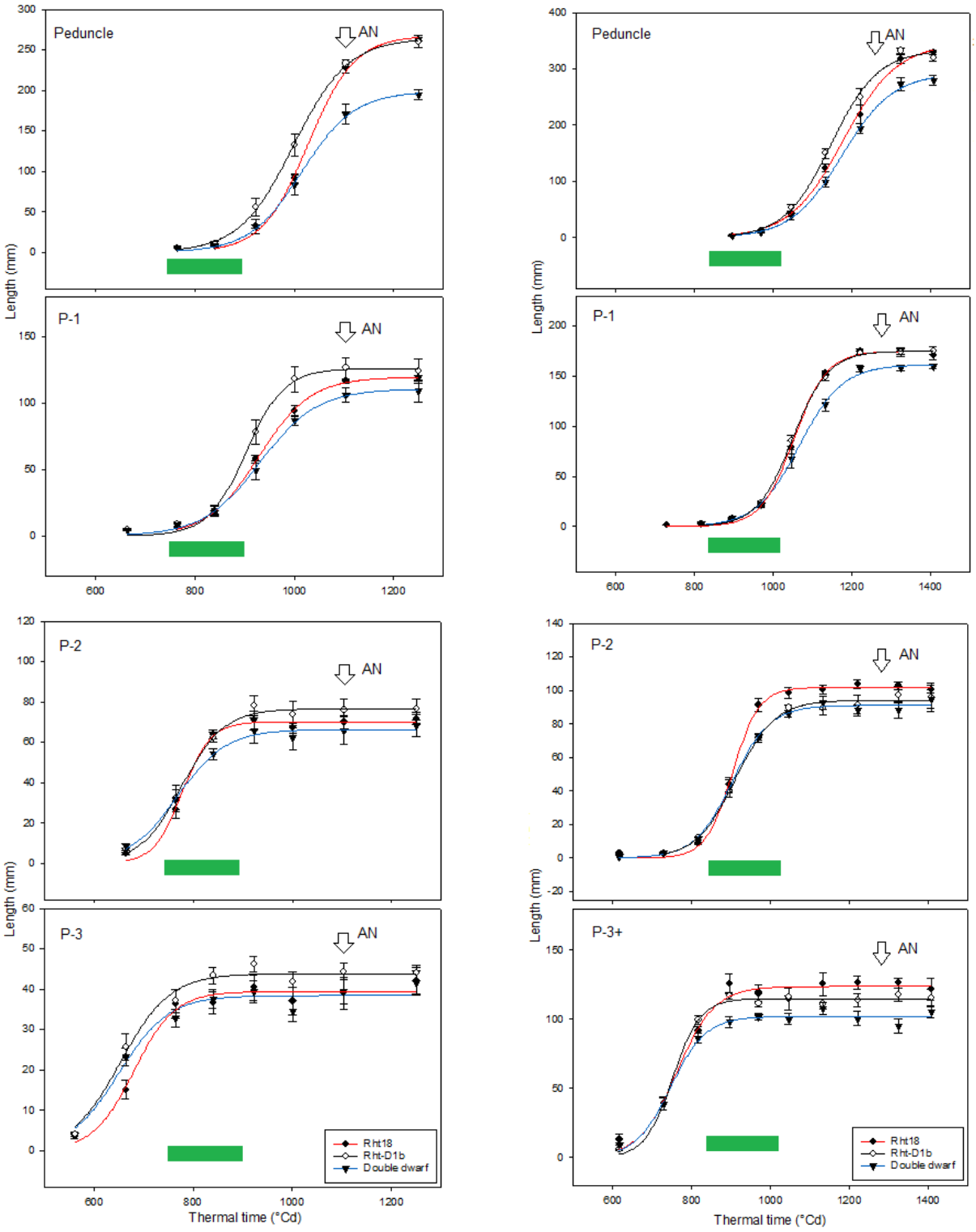


Figure 4.3 Change in internodes length over time in Expt 1 (left column) and Expt 3 (right column). Error bars represent the standard error. AN means anthesis. Curves were fitted in a 3 parameter sigmoid model in SigmaPlot (Ver. 12), red, black and blue curves indicate *Rht18*, *Rht-D1b*, and Double dwarf respectively. Green bar indicates MGPS in length. Tall genotype was excluded in the figure to give more resolution between lines with the dwarfing genes.

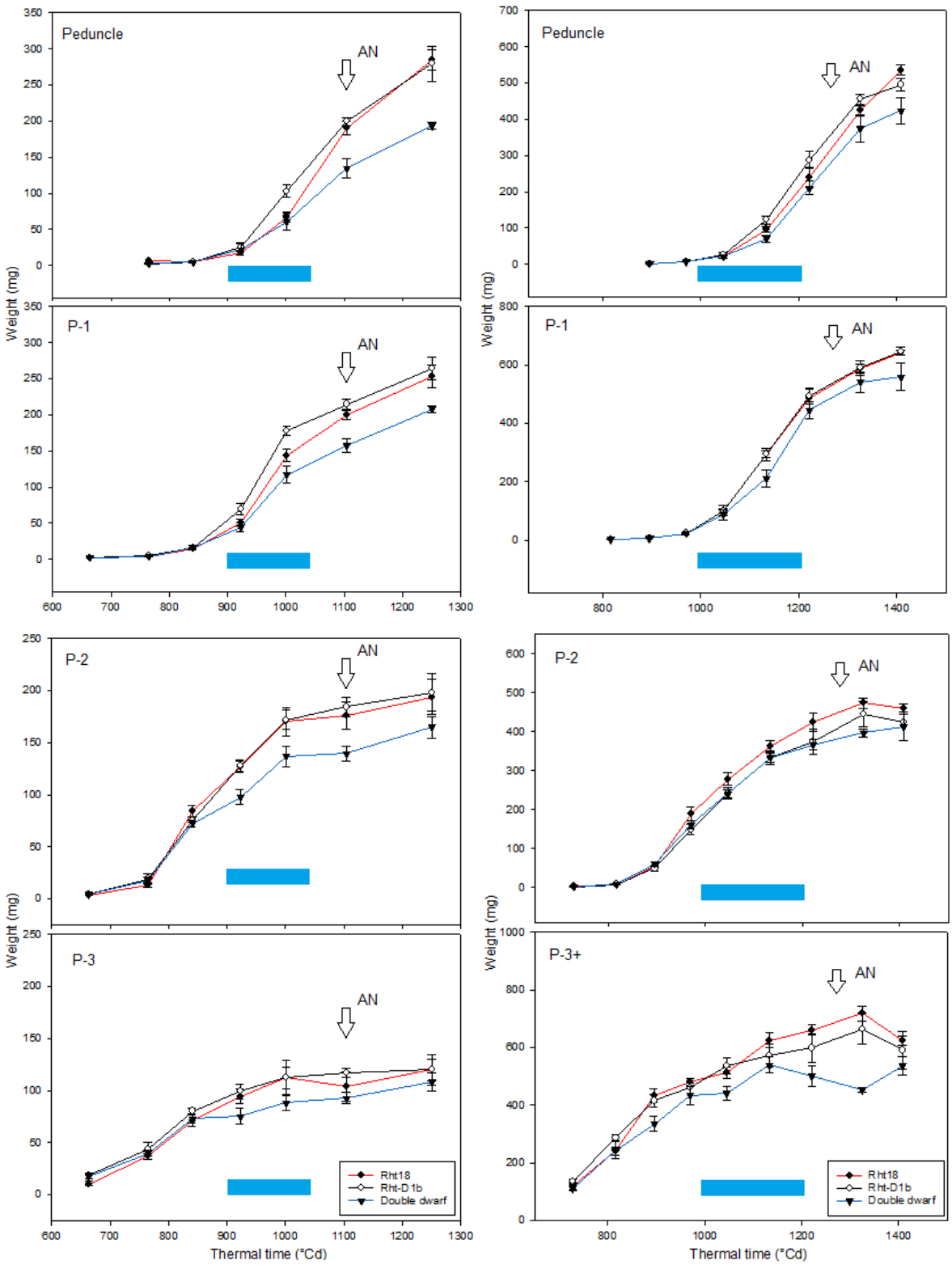


Figure 4.4 Change in internodes weight over time in Expt 1 (left column) and Expt 3 (right column). Bars represent the standard error. AN means anthesis. Red, black and blue lines indicate *Rht18*, *Rht-D1b*, and Double dwarf respectively. Blue bar indicates MGPS in weight. Tall genotype was excluded in the figure to give more resolution between lines with the dwarfing genes.

The beginning of peduncle elongation was later in *Rht18* and Double dwarf than in *Rht-D1b* in Expt 1 and in Expt 3 (Figure 4.3). However, by anthesis the peduncles had the same length, and this suggests a delay in the beginning of stem elongation in *Rht18*. This is also evident in stem elongation of Expt 1 (Figure 4.1). The difference was reduced in Expt 3, but the trend was still visible. As expected, the Double dwarf was shorter than semi-dwarfs before anthesis. At P-1 internode, *Rht18* still showed the later initiation than *Rht-D1b* in Expt 1, but not in Expt 3. In Expt 3, three genotypes stayed very close until Tt=1046 °Cd, single dwarfs had faster growth rates and the lengths were longer than Double dwarf at the next sampling point. The growth curve of *Rht18* overlapped with *Rht-D1b* during the sampling period with narrow error bars. Three genotypes were similar at P-2 in Expt 1, but *Rht18* stood out from *Rht-D1b* and Double dwarf at Tt=970 °Cd in Expt 3 suggesting a higher growth rate from Tt=896 °Cd to Tt=970 °Cd, which coincided primarily with MGPS in length. For internodes below P-2, the differences between *Rht18, Rht-D1b* and Double dwarf were not consistent.

The weight data was similar to length data before anthesis. The peduncle of *Rht18* was significantly lighter than *Rht-D1b* but heavier than Double dwarf approximately 100 °Cd before anthesis in both populations, but after anthesis, *Rht18* caught up with *Rht-D1b* and ended up with similar weight. For the P-1 internode, *Rht18* delayed in increasing weight but was similar to *Rht-D1b* in Expt 1 after anthesis, while the two semi-dwarfs had identical weight accumulation in Expt 3. *Rht18* could not be distinguished from *Rht-D1b* in both populations for the P-2 internode, but both genotypes were distinguished from Double dwarf in Expt 1.

No difference in anthesis date was found between *Rht18* and *Rht-D1b* (Table 2.6, Chapter 2) indicating that phenology was similar across genotypes. Before anthesis, however, *Rht18* had overall less spike weight than Tall or *Rht-D1b* in both populations (Table 4.2), and the peduncle growth rate was found to be slower than *Rht-D1b* in both populations,

suggesting this gene may affect growth rate of distal internodes and spike before anthesis. The data from Expt 4 (Table 4.3) supported the result from Expt 1 and Expt 3, *Rht18* had the shortest peduncle at booting but it overtook Double dwarf at anthesis and showed no difference to *Rht-D1b* when it ceased elongation. Similarly P-1 internode, *Rht18* was 26% shorter and 29% lighter compared with *Rht-D1b*, and the length and weight gaps were reduced to 8.5% and 11% at anthesis.

4.3.1.4 Change of spike stem index pre-anthesis

The development of spike stem index (SSI), measured as the ratio of ear weight to total weight of ear plus stem, from terminal spikelet to post-anthesis can be separated into three stages. The first stage where there was a linear increase in SSI that corresponded to a period from early stem elongation to the time of heading (approx. Tt=350 °Cd) (Figure 4.5). The second stage was from heading to anthesis where there was little increase in SSI. The third stage was post-anthesis where SSI began to increase again. During the first stage spike length increased together with lower internodes such as P-2 and P-3, and SSI increased linearly, thus demonstrating a preferred carbon allocation to the spike than to the stem. This was greater in lines with the dwarfing genes than it was with the tall lines in both Expt 1 and Expt 3. The Double dwarf had a slightly higher SSI than other genotypes in Expt 3. In the second stage from heading to anthesis the SSI plateaued suggesting equal allocation of carbon to both the spike and the developing internodes, principally P-1 and the peduncle. This demonstrates the stronger sink for assimilate in the stems than to the developing ears than before heading. The same results were found in both Expt 1 and 3. Dwarfing genes continued to have their SSI advantages over Tall and the Double dwarf exceeded semi-dwarfs around anthesis in Expt 1. In Expt 3, the ranking of SSI was clear: Double dwarf > *Rht-D1b* > *Rht18* > Tall due to the late spike development in *Rht18*. In the third phase after anthesis, spike growth was boosted

relative to stem growth due to the grain formation and development, which resulted in an increasing SSI after anthesis. The genotypic trend was kept after anthesis in both Expt 1 and Expt 3.

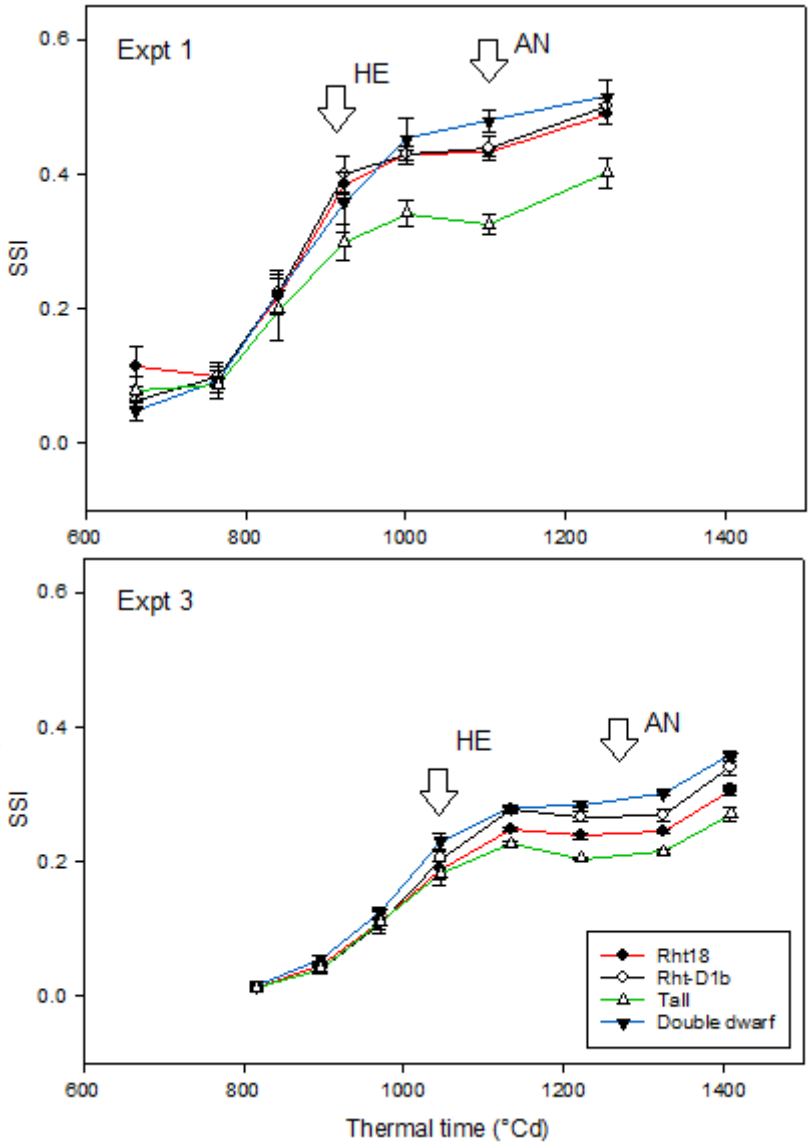


Figure 4.5 Change of spike stem index before anthesis in Exp1 and 3. HE and AN refer to heading and anthesis respectively.

4.3.2 Post-anthesis stem-stored dry matter remobilisation to grain growth

4.3.2.1 Change of dry weight in stem and each internode

By anthesis, stems had reached their full length, however, dry matter accumulation in the stems continued until a maximum was reached approximately 200 °Cd after anthesis (Figure 4.6). This maximum value was maintained for a longer duration in Expt 3 but not in Expt 1 before it declined. Most of this increase in dry weight in the stem may be due to storage of WSC as stem weight declined thereafter to values similar to that at anthesis in Expt 1 but to values lower than those at anthesis in Expt 3 (Figure 4.6). On average, about 30% of the stem weight was lost by maturity.

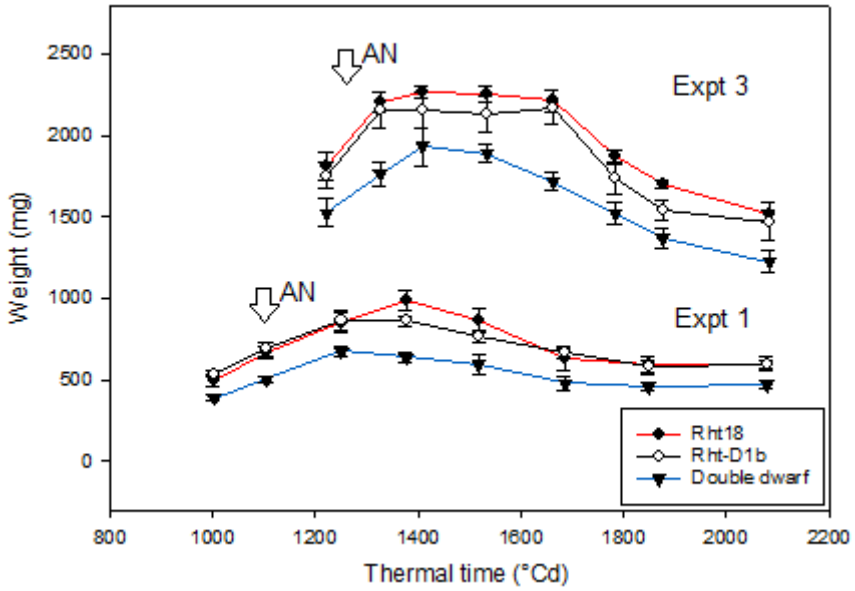


Figure 4.6 Dry weight changes in stem for *Rht18*, *Rht-D1b* and Double dwarf in Expt 1 and Expt 3 populations after anthesis. AN: anthesis, lower and upper graph represent Expt 1 and Expt 3 respectively.

In both Expt 1 and Expt 3, *Rht18* had significantly larger maximum stem weights than *Rht-D1b*, but ended up with the same weight at maturity (Figure 4.6). The loss of stem dry matter was calculated by the subtraction between stem weights from sampling times with

maximum stem weights (averaged of samplings 1, 2, 3 after anthesis in Expt 1, and 1, 2, 3, 4 after anthesis in Expt 3) and maturity (averaged of the last two samplings in Expt 1 and the last one in Expt 3) (Table 4.4). Stem and internodes dry matter change differed among experiments. The amount of dry matter relocated in total stem ranked as Tall > *Rht18* > *Rht-D1b* > Double dwarf, but the difference between Tall and *Rht18* was not significant. The genotypic difference in relocated dry matter was gradually reduced at each internode from top to bottom. Internode P-1 lost the most assimilates amongst the distal three internodes followed by P-2 and then peduncle, suggesting loss of dry matter of each internode can be affected by linear density rather than length.

As expected, differences in the contribution percentage of internodes were found between Expt 1 and Expt 3 mainly due to the difference in grain weight driven by different sowing time. No difference was found at genotypic level for spike weight increase regardless of the big influence from different experiments (Table 4.4). No differences were found in any internode contribution among *Rht18*, *Rht-D1b* and Tall. There was a significant difference between double dwarf lines and other genotypes in terms of whole stem, and this difference was mainly contributed by the difference from the peduncle. Double dwarf lines showed an additive effect on contribution percentages of internodes from both semi-dwarfing genes, particularly on peduncle.

Table 4.4 Means of decrease in dry matter and loss as percentage of grain dry matter increase in stem and internodes in Expt 1 and Expt 3. The interaction between experiment and genotype was not significant. (Abbreviation: Treat, treatment; *RD1b*, *Rht-D1b*; DD, Double dwarf; SWI, spike weight increase; Ped, peduncle)

Treat	SWI (mg)	Stem (mg)	Ped (mg)	P-1 (mg)	P-2 (mg)	P-3+ (mg)	Stem %	Ped %	P-1 %	P-2 %	P-3+ %
Genotype											
Rht18	1390	509	106	161	129	113	38.5	8.5	12.8	10.0	7.1
RD1b	1408	465	86	161	121	98	34.8	7.1	12.4	9.2	6.0
Tall	1512	556	123	176	127	130	37.3	8.9	12.1	9.0	7.2
DD	1345	400	64	137	110	89	28.8	4.9	10.0	7.9	5.9
l.s.d.	ns	78***	29***	25*	ns	ns	6.8*	2.3 **	ns	ns	ns
Environment											
Exp 1	766	262	63	94	71	33	35.3	8.6	12.8	9.7	4.3^
Exp 3	2062	703	126	223	172	182	34.4	6.1	10.9	8.4	8.9
l.s.d.	72***	55***	20***	18***	8***	27 ***	ns	1.6 **	1.8*	ns	1.5 ***

*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$, ns: not significant, ^: plants in Expt 1 have one less internode below P-2

4.3.2.2 Difference in linear density for each internode

The changes in linear density of internodes showed the extent of dry matter mobilised per unit stem length. The linear density of the distal three internodes was compared between *Rht18* and *Rht-D1b* from anthesis to maturity in Expt 1 and Expt 3. The pattern of change in linear density for each internode was very similar (Figure 4.7). The linear density of *Rht18* was significantly greater than *Rht-D1b* in Expt 1, but not in Expt 3 showing that the environment has a significant impact on this trait. The changes in internode linear density were compared at genotypic level combining two experiments (Table 4.5) using the approach described for dry matter loss in Section 4.3.2.1. Dwarfing genes remobilised more dry matter than Tall and there were no differences between *Rht18* and *Rht-D1b* in the distal three internodes.

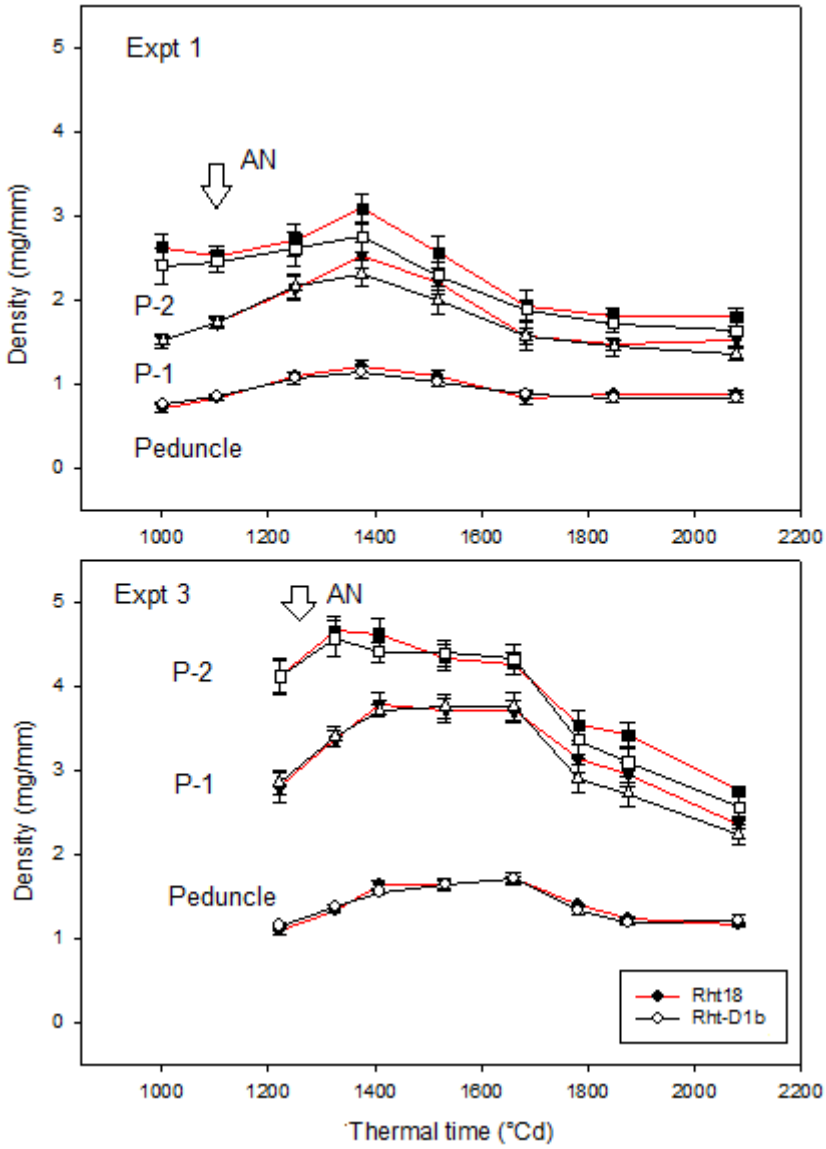


Figure 4.7 Changes of density in distal three internodes over time in *Rht18* and *Rht-D1b* in Expt 1 and Expt 3 populations

Table 4.5 Means of change in linear density (mg mm^{-1}) after anthesis for distal three internodes averaged in Expt 1 and 3.

Genotype	Peduncle	P-1	P-2
Rht18	0.38	1.08	1.32
Rht-D1b	0.33	1.13	1.34
Tall	0.31	0.87	1.03
Double dwarf	0.31	1.00	1.23
l.s.d.	ns	0.19*	0.2*

*: $P<0.05$, ns no significance

4.3.2.3 Post-anthesis change in spike stem index

The SSI was calculated for Expt 1, Expt 3 and Expt 4 after anthesis. Expt 4 has the similar genotypic result to Expt 1 and Expt 3, which can be ranked as Double dwarf > *Rht-D1b*= *Rht18* > Tall (Table 4.3). At the second sampling in Expt 4, *Rht18* showed lighter spike and distal internodes such as peduncle and P-1 compared with *Rht-D1b*, but the difference disappeared at maturity. This delay did not result in a difference in SSI. From booting to one week after anthesis, differences in SSI were reduced between semi-dwarfs and talls but increased between semi-dwarfs and double dwarfs, suggesting the significant additive effect of *Rht18* and *Rht-B1b* for this trait.

4.3.3 Fruiting efficiency

Fruiting efficiency (FE) known as grains set per unit of spike dry weight at anthesis did not show any significant differences between different genotypes (Table 4.6). *Rht18* had higher FE than *Rht-D1b* and Tall across three experiments, which was due to smaller spike at anthesis, while grain number was similar to other genotypes (see Table 2.7, Table 2.8 and Table 2.9). Double dwarf was affected by *Rht18* in Expt 1 particularly, but not in other two experiments.

Table 4.6 Summary data for means of fruiting efficiency (grains g spike-1) at anthesis in Expt 1, Expt 2 and Expt 4.

	Expt			
Genotype	1	3	4	Mean (Genotype)
Rht18	94.8	63.7	80.8	79.7
Rht-D1b	85.5	59.7	77.1	74.1
Tall	88.3	56.1	70.5	71.7
Double dwarf	107.6	57.2	71.2	78.7
Mean (Expt)	94.1	59.2	74.9	

l.s.d. was 8.3ns for Genotype, 7.2*** for Expt and 14.3ns for Genotype × Expt. ns: not significant, ***: $P<0.001$

4.4 Discussion

Previous chapters have demonstrated that there is a potential advantage in *Rht18* over the current dwarfing genes with longer coleoptiles, and the importance of this where deep sowing is required and if soils are warm (Chapter 3). Grain yield traits revealed no penalty associated with *Rht18* compared to *Rht-D1b/B1b* (Chapter 2). The results presented in this chapter show that *Rht18* is virtually indistinguishable from the current GA insensitive dwarfing genes in detailed aspects of development and growth. This is despite the different mechanism of dwarfing i.e. *Rht-B1b* and *Rht-D1b* are insensitive to GA whereas *Rht18* is sensitive to GA.

The time of anthesis in lines with or without *Rht18* is mostly the same as is the case for the current major dwarfing genes (see Chapter 2). However, there was some evidence that earlier developmental stages could be delayed in *Rht18* lines. For example, *Rht18* lines in the serial harvests had shorter spike lengths and stem lengths during early vegetative growth than other lines but they caught up by anthesis (Figure 4.1). This was also true for internode lengths (Figure 4.3) and weights (Figure 4.4). It was more evident in Expt 1 than Expt 3 and so it may be associated with the greater genetic diversity between lines in Expt 1. This may also be attributed to the difference in planting date between the two experiments. Nevertheless, it was also evident in the field experiment (Expt 4) where the spike weight and peduncle length of *Rht18* lines were less than lines with *Rht-D1b* during the early pre-anthesis phase (Table 4.3). By anthesis, the growth of both spike and internodes of *Rht18* caught up with Tall or *Rht-D1b* in Expt 4 and finally the genotypic difference was negligible. To determine more precisely whether there was a difference in development between *Rht18* and other lines more careful examination of development is required.

According to Waddington et al. (1983), spike length is correlated with spike developmental score in barley, thus early spike (apex) length from the first sampling time can be used to represent the apex stage in wheat. No genotypic difference for spike length at TS

was found in both experiments (Table 4.1), which suggests that *Rht18* and *Rht-D1b* have equivalent spike developmental stages at the beginning of stem elongation. According to Fischer and Stockman (1986) and Richards (1992a) dwarfing genes such as *Rht-D1b* may have a longer duration from initiation to anthesis than the tall wheats, and double dwarfs may even have a further delay. However, the spike length at terminal spikelet reported here suggests *Rht-D1b* did not delay spike development compared with Tall, neither did *Rht18* nor Double dwarf. Thus, the delayed growth of *Rht18* in spike and distal internodes is perhaps the result from longer late developmental periods rather than a difference in floral initiation. On the other hand, floral initiation study from durum wheat suggests *Rht18* lines have a significantly earlier floral stage than tall lines (data not shown), which contrasts with the TS spike length result. *Rht12* showed significant delay in apex development as early as double ridges (DR) (Chen et al. 2013). Therefore, further studies targeting floral development from DR to anthesis should be conducted in this population to understand if *Rht18* had impact on early spike and internode development.

The competition between spike and stem growth was studied during the critical period defined from terminal spikelet to anthesis (Kirby 1988). Gonzalez et al. (2011) confirms the pre-anthesis competition theory based on the finding of the strong positive association between number of fertile florets or grains and spike dry weight at anthesis and floret death occurs at maximum spike growth. It was suggested that heavier spike at anthesis means more dry matter partitioned for spike growth, which leads to more fertile florets or grains thus less competition in dwarfing genes. This proposal was supported earlier by Fischer and Stockman (1986) and Miralles et al. (1998b) who had found the semi-dwarf cultivars containing *Rht-B1b* or *Rht-D1b* reduced stem competition with spike by partitioning less dry matter to stem growth to exhibit a larger number of grains than the Tall counterpart. *Rht18* showed a higher ratio of spike/biomass and lower ratio of stem/biomass compared with tall parents at anthesis in three backgrounds (Yang et al. 2015). In this study, SSI was plotted from around terminal

spikelet to just after flowering. From the beginning of stem elongation there was greater carbon allocation to the growing spike up until heading. Just after heading to anthesis, there was equal allocation of dry matter to spikes and stem (Figure 4.5). This latter period coincides with the most rapid growth of late internodes P-1 and the peduncle. The spike also continues to grow up to anthesis but it cannot out-compete the peduncle plus P-1 for resources. There are genetic differences in the ability to compete that is attributed to the dwarfing genes. The tall lines are least able to compete and the double dwarf lines are most competitive. *Rht18* and *Rht-D1b* are equally competitive. This raises the question whether the length of the peduncle could be shortened, relative to other internodes (Richards 1996). There is evidence that *Rht13* may have shorter peduncle and hence reduce competition to the growing spike (Rebetzke et al. 2011). Another advantage of *Rht13* is that most of the peduncle is enclosed by the flag leaf sheath and so may also have higher carbohydrate storage as the exposed peduncle does not store WSC (Scofield et al. 2009).

According to Gonzalez et al. (2011), maximum spike growth was associated with the onset of floret death. To better understand the competition between spike and stem during the critical period, the MGSP capturing 80% of the spike growth was compared with all internodes to determine which internode overlaps with spike growth and whether *Rht18* behaves differently compared with Tall or *Rht-D1b*. Internodes grow sequentially from basal to distal, and the number of internodes for main stem can vary when growing in different conditions. In this study, plants sown in winter (Expt 3) had one more internode than those sown in spring (Expt 1), thus the distal three internodes peduncle and P-1, P-2 and P-3 were the most consistent internodes to compare. It is thought that the MGSP mainly coincides with the growth of the peduncle (Kirby 1988). However, this was only partly observed in the experiments conducted in this study. Here the MGSP in weight coincided with the growth of P-1, P-2 and only partly the peduncle. This suggests that P-1 and P-2 are also important in terms of the competition between spike and stem. As previously reported, the start of floret

death coincides with rapid extension of peduncle (Siddique et al. 1989). In the study from Kirby (1988), growth of internodes was not monitored after anthesis, and growth of peduncle continues post-anthesis thus the maximum time of peduncle might be misjudged. However, this chapter did not look at the floret death which could occur within the period when both P-1 and peduncle grow rapidly. A further experiments would be to evaluate the relationship between maximum spike and internode growth and the onset of floret death.

Stems grow in weight both before anthesis and for approximately 2-3 weeks after anthesis after the end of stem elongation. Part or all of the later increase in weight could be due to the temporary storage of WSCs. Substantial losses of dry weight from the stems then occurred during the latter half of the grain filling stage. About one third of this loss was found to be due to stem respiration and the rest to mobilisation to grains (Rawson and Evans 1971), although this finding was from glass house grown plants.

Consistent with the result from Borrell et al. (1993), the absolute amount of mobilised dry matter (mg/stem) from stems was reduced in *Rht-D1b*, *Rht18* and Double dwarf under non-water stressed condition. Internode P-1 had the greatest loss in dry matter rather than the peduncle, even though both have the same weight. This is likely to be due to the lack of stored carbohydrates in the peduncle which is not surrounded by the leaf sheath (Scofield et al. 2009). This is also consistent with the result from Cruz-Aguado et al. (2000) who hypothesised that depletion of dry matter from peduncle can be compensated by photosynthesis of the exposed peduncle and import of current assimilate from the flag leaf and P-1 during grain filling, which was more than the compensation to internode P-1. Thus, P-1 internode supplied a larger amount of non-structural carbon than other internodes for grain filling. Means of relocated dry matter relative to specific weight of each internode were calculated in this research, and the result showed that semi-dwarfs contributed higher dry matter per unit length than Double dwarf and Tall, suggesting dwarfing genes are more efficient in storing and then relocating the assimilates than Tall. Interestingly the averaged

linear density of the distal three internodes over the period was ranked as Double dwarf < Semi-dwarfs < Tall (data not shown), which is different to that reported by Borrell et al. (1993) who found that specific weight in *Rht-B1b* or *Rht-D1b* was not different from the Tall. So the result from this study suggested that semi-dwarfing genes relocate more assimilates per unit length but not as an internode or whole stem compared with the Tall. *Rht18* behaves very similar to *Rht-D1b* in specific weight except in Expt 1 where presumably *Rht18* translocated more carbohydrate than *Rht-D1b*.

Relocated dry matter from stem can amount to as much as 25-40% of the grain growth during the same time interval and it is also called contribution of stored carbon to grain yield (Blum 1998). The semi-dwarfs had the same contribution as Tall, and Double dwarf had a significant 23% reduction compared with Tall as a result of additive effects. According to Shakiba et al. (1996), lines with *Rht-B1b* or *Rht-D1b* have lower yield contribution from stem than Tall under irrigated conditions, but they may exhibit higher contribution under dry conditions. No difference was found between *Rht18* and *Rht-D1b* in this study, and it will be interesting to test *Rht18* in non- irrigated trials for the contribution to grain yield. However, as previously reported that spike growth could be limited by sink (capacity of grains to accumulate assimilates) rather than source (Savin and Slafer 1991), in that the yield is more associated with germplasm or environments which favour many grains and growing individual grain mass while less rely on the source for assimilates.

4.5 Conclusion

Lines with *Rht18* were shown to be very similar to lines with *Rht-D1b* in three field experiments varying markedly in sowing time and crop duration. Early dry matter of the stem and spikes were similar as was the change in stem dry matter, which reflects storage and remobilisation of dry matter from the stem. However, both *Rht18* and *Rht-D1b* varied from

the Tall and the Double dwarf. Thus, semi-dwarfing genes behaved in a very similar way and most of the variation was attributed to variation in height and not specifically to the genes themselves. This is a similar conclusion to Richards (1992a) when reporting on *Rht-B1b* and *Rht-D1b* compared with Tall and Double dwarf. Some differences were noted, for example, it seems as if *Rht18* may delay the beginning of stem elongation and spike growth more than *Rht-D1b*, although no differences were detected by anthesis. There was also some evidence that *Rht18* may store and remobilise more stem carbohydrates than equivalent *Rht-D1b* lines.

Chapter 5 Fine mapping *Rht18* in durum wheat

5.1 Introduction

Wheats with dwarfing genes can be easily selected by breeders using the reduced height phenotype. Conventional semi-dwarfing genes such as *Rht-B1b* or *Rht-D1b* reduce plant height by about 20-25% (Richards 1992a; Flintham et al. 1997). As described in the physiological study in chapter 2, *Rht18* is about the same height as *Rht-D1b* or *Rht-B1b*, reducing height by approx. 25% compared with tall plants. Thus, selection of plants that carry one or the other gene in populations that segregate for both *Rht18* and *Rht-D1b/B1b* is difficult through phenotype alone. Molecular markers are an important selection tool for traits where phenotypic differences are difficult to detect. Molecular markers are based on DNA patterns which are not affected by environment. Markers can be examined during all stages of development and recessive alleles can be detected without the need for progeny testing. For *Rht18* to replace *Rht-D1b/B1b* in breeding, it will be critical to have molecular markers available for both genes.

Rht18 was discovered as a mutation in the tall durum wheat 'Anhinga', induced by fast neutron radiation and released as cultivar 'Icaro' (Konzak 1987). Durum wheat is tetraploid, comprising two genomes (A and B) compared with hexaploid wheat with three genomes (A, B and D). Genetic mapping and marker development is therefore easier in tetraploid wheat. *Rht18* was previously mapped on chromosome 6AS and was linked to microsatellite or simple sequence repeat (SSR) marker *BARC3* (Haque et al. 2011). However, the *BARC3* marker was only tested in a small number of F_2 lines that were selected for Bulk Segregant Analysis (BSA) and not in the larger F_2 mapping population. Furthermore the height classification of F_2 lines was ambiguous without progeny testing. Therefore, additional

genetic mapping of *Rht18* was necessary to find a tightly linked, diagnostic marker which can be used by breeders in marker assisted selection.

SSR polymorphism is abundantly distributed in the wheat genome. SSR markers comprise short repeat units of 1-6 nucleotides, and polymorphism can be displayed via length variation using primers flanking the simple repeated region (Tautz and Renz 1984). It requires high resolution gels to discriminate allele size differences, which is expensive and time consuming. The next generation of molecular markers is based on single nucleotide polymorphism (SNP) which are abundant and easy to assay using high throughput, gel-free genotyping platforms (Brookes 1999). The technique involves the identification of single base pair changes at specific sites in the genome and the development of PCR-based protocols to assay the polymorphism. For example, *Rht-B1b* and *Rht-D1b* gene-based markers were developed after these genes were isolated and single base pair mutations identified (Peng et al. 1999). These mutations were targeted by Ellis et al. (2002) for primers designed to discriminate the SNP polymorphisms. Thus, markers based on this SNP information are now being used to select for these genes across a wide range of germplasm in many different breeding programs. Discrimination of SNP alleles can be detected using competitive allele-specific assays involving different fluorophores in the PCR reaction, with products viewed through a plate reader which can measure fluorescence. One of the commercial SNP platforms is the KASPar^{TM} genotyping assay.

SNP markers can be assayed using automated genotyping platforms that allow the screening of a large number of markers and thousands of lines simultaneously (Ganal et al. 2014). The SNP genotyping platforms were enabled by the manufacture of DNA arrays with oligonucleotide probes bound on solid surfaces which can screened by hybridising genomic DNA (McGall and Christians 2002) or using single base primer extension to determine specific alleles (Steemers et al. 2006). Large SNP arrays have been established in crops, such as the 4.4 K SNP array in rice (*Oryza sativa*) (Zhao et al. 2011) or the 50K array in maize

(*Zea mays L*) (Ganal et al. 2011). In wheat, a 9K array was first generated which was then increased to a 90K array that contained SNPs from both hexaploid and tetraploid wheats of diverse geographical origin (Cavanagh et al. 2013; Wang et al. 2014). The 90K SNP array was used to construct genetic linkage maps using several mapping families that were later combined to construct a consensus linkage map for wheat (Maccaferri et al. 2015).

Although there are close to 90,000 SNPs on the DNA array, these may not be polymorphic in specific crosses and may not cover some genomic regions very well. It is therefore sometimes necessary to identify additional SNPs within target populations or genomic regions. The 'Genotyping by Sequencing' (GBS) approach utilises rapid advances in next generation sequencing technologies to generate and map SNPs in specific mapping populations or in diversity studies (Poland and Rife 2012). Another approach to identify new markers is to utilise the wheat genome sequence which is being generated as part of the International Wheat Genome Sequence Consortium. A physical map of chromosome 6A of Chinese Spring wheat cultivar has recently been completed using individual BAC clones that were fingerprinted to build contigs that were anchored to the genetic map (Poursarebani et al. 2014). Some of these contigs have already been sequenced, and these sequences can now be utilised for generating additional markers in the *Rht18* region.

The objectives of this study were to (i) fine map *Rht18* in tetraploid wheat using recombinant inbred lines (RILs) as a mapping population, and (ii) develop SNP markers that can assist in replacing *Rht-B1b/D1b* with *Rht18* in commercial breeding programs. Before tightly linked markers are useful to breeders, the 'background' frequency of the allele to be introgressed needs to be assessed in a wide range of germplasm. Ideally the marker allele associated with the gene to be introgressed will be unique.

5.2 Material and methods

5.2.1 Mapping population

A durum mapping population was generated by intercrossing the dwarf mutant Icaro (*Rht18*) and the tall cultivar Langdon. In the F_2 progeny, a homozygous short and a homozygous tall plant were selected and intercrossed. Approximately 200 F_2 half seeds were screened with flanking SSR markers *BARC3* and *GWM356* to develop recombinants in the genetic interval. With one additional SSR marker *WMS4608* identified in this region, recombinant F_2 lines were fixed for three markers and advanced to F_4 or F_5 generation by single seed descent. Finally the mapping population consisted of 39 recombinant inbred families, with 24 short and 15 tall lines.

5.2.2 Phenotyping

Plant height was measured when stems stopped elongating, and length was recorded from the soil surface to the top of ear. Plant height was measured in rows at three different positions and the average recorded.

5.2.3 Identification of chromosome location

Chromosome location of *Rht18* was previously identified by BSA using multiplex ready PCR technology (Hayden et al. 2008). Linked SSR markers were identified using DNA bulks from short and tall progenies as well as from short and tall parents. SNP markers were identified using the same approach by screening DNA bulks on the 9K and 90K DNA arrays. DNAs from 10-12 lines of the same phenotype were grouped for each bulk.

5.2.4 Genotyping

5.2.4.1 DNA extraction

DNA was extracted from freeze-dried tissue in deep-well microtitre plates following the procedure described in Ellis et al. (2005). A NanoDrop® ND1000 spectrophotometer (Thermo Fisher Scientific Inc, USA) was used for quantification of DNA samples.

5.2.4.2 SSR assay

Primer sequence for SSR marker *BARC3, GWM356* was obtained from http://wheat.pw.usda.gov/ and *WMS4608* from Traitgenetics, GmbH, Germany. Sequences of four primers are listed in Appendix Table 5.1.

PCR was carried out in a S1000 thermal cycler (Bio-Rad) using denaturation at 94°C for 4 min followed by 15 touchdown cycles of 30 sec at 94°C, 30 sec at 65-50°C (decreasing 1°C per cycle) and 45 sec at 72°C. Another 30 amplification cycles consist of 15 sec at 94°C, 15 sec at 50°C and 45 sec at 72°C. One additional step was performed at 72°C for 5 min for final elongation of the PCR product. Each PCR reaction of 10 µl contained 50–100 ng of template DNA, 2µl of 5×GoTaq buffer (Promega), 0.1µl GoTaq polymerase enzyme (Promega), 0.2 µl, 1 µl, and 1 µl for 5µM forward, reversed and M13 primers which is tagged with different fluorescences (FAM, VIC, NET and PET), 250 µM each dNTPs and 6 µl Milli-Q H_2O.

PCR products were run on 1.5% agarose gels stained with ethidium bromide to check the concentration and 2-3 µl of each sample was diluted 50-100 times to achieve the optimum detection range of DFA (DNA Fragment Analyzer). Each sample was mixed with 10 µl HiDi and 0.1 µl Liz500 (standard) and denatured at 94°C for 4 min. Samples were then injected by 3130XL Genetic Analyzer (Applied Biosystems, HITACHI) and data analysed by GeneMarker V1.95 to determine allele size.

5.2.4.3 KASPar assay

Primer design: Sequences containing the SNP which is linked with *Rht18* from 9K and 90K DNA arrays were used to design primers by online software Primer3: WWW primer tool. Each set of primers consists of a pair of allele-specific primers (one for each SNP allele) and one common primer.

A KASPar reaction of 8µl consists of 0.11 µl 72 × assay mix (formulation of 3 primers in certain ratio) and 4 µl reaction mix (containing Taq polymerase enzyme and the passive reference dye, ROX, $MgCl_2$ and DMSO) with 4 µl template DNA (1-40 ng/µl). Details of KASPar reaction mix can be found at http://www.lgcgroup.com. DNA amplification conditions were as follows: denaturation at 94°C for 4 min followed by 10 touchdown cycles of 20 sec at 94°C and 60 sec at 65°C (decreasing 0.8°C per cycle). Another 32 amplification cycles consisting of 20 sec at 94°C and 60 sec at 57°C. After amplification the plate was placed in fluorescent reader and genotypes were scored by allele discrimination function of Bio-Rad CFX manger 3.0.

5.2.5 Genetic map construction

The 39 mapping families were genotyped for 3 SSR and 10 SNP markers and genetic distance (cM) was calculated by counting the recombination events that were detected in 400 gametes derived from 200 F_2s.

5.2.6 Sequencing assay

The primers of *WMS4608* were used to amplify fragments in both Chinese Spring and nulli-tetrasomic (N6AT6B) of Chinese Spring to find if the marker is genome specific (on

chromosome 6A). The marker was then sequenced from Chinese Spring using the sequencing protocol below.

Sequencing protocol consisted of four steps: 1. PCR, 2. PCR product cleaning, 3. PCR for sequencing (one way) and 4. Cleaning for sequencing.

1. PCR reaction of 20 µl consists of 100 ng of template DNA, 4µl of 5×GoTaq buffer (Promega), 0.2µl GoTaq polymerase enzyme (Promega), 1 µl for 10µM forward and reversed primers respectively, 250 µM each dNTPs and 10 µl Milli-Q H_2O. The amplification process using denaturation at 94°C for 4 min followed by 5 touchdown cycles of 30 sec at 94°C, 30 sec at 64-60°C (decreasing 1°C per cycle) and 80 sec at 72°C. Another 32 amplification cycles consist of 15 sec at 94°C, 15 sec at 60°C and 45 sec at 72°C.

2. PCR product was purified using Exosap-IT (Affymetrix) treatment and incubating at 37°C for 30 minutes followed by 10 minutes at 95°C to terminate the reaction.

3. PCR for sequencing was performed in a reaction mix: 3 µl cleaned PCR product, 2 µl 5×sequencing buffer, 2 µl 10 µM primer (one way), 2 µl Big Dye V3.1 and 3 µl MilliQ water. Sequencing reactions were carried out as pre-heat at 94°C for 4 min followed by 30 cycles of 94°C for 10 sec, 50°C for 5 sec and 60°C for 4 min, followed by 60°C for 5min and 25°C for 5min.

4. Sequencing reaction product (12 µl) was added to 3 µl of 3M sodium acetate (pH 4.6-5.2) with 8 µl Milli-Q. The reaction mix was precipitated in 40 µl of 100% ethanol at room temperature for 15 min then centrifuged at 13,000 rpm for 15 min. The supernatant was removed and 250 µl of 70% ethanol was added to each sample, mixed well, and then centrifuged at 13,000 rpm for 10 min. Finally, the supernatants were removed and pellets were dried.

Selected genes or fragments from the 8 Mb contig were sequenced as described above. Primers were designed by Primer3: WWW primer tool. Analysis of sequencing reaction

products were carried out at John Curtin School of Medical Research (JCSMR), ANU and data was viewed and edited by FinchTV v.1.4.0.

5.3 Results

Previously, *Rht18* was mapped between SSR markers *BARC3* and *GWM356* using a mapping population derived from the Icaro × Langdon cross. Additional SSR marker *WMS4608* was mapped and showed tight linkage to *Rht18*. Two progeny lines, one short line carrying *Rht18,* the other tall were used to generate a second population. 200 F_2s were screened with the three SSR markers to identify 39 lines that carried recombination events. These 39 lines were used to fine map *Rht18* as part of this project.

5.3.1 Phenotyping of *Rht18* using homozygous recombinant lines

The 39 lines were planted as F_3 or F_4 rows in the birdcage and their height was measured at maturity. Mean height of the short parent was approx. 95 cm, and the mean height of the tall parent was approx. 130 cm. There were 21 lines that were uniform and less than 110 cm tall, these lines were classified as homozygous short which were likely to carry *Rht18*. In addition, seven lines which were uniform and taller than 120 cm were classified as tall because they were unlikely to carry *Rht18*. There were three lines uniform in height that could not be classified due to their intermediate height (between 110 cm and 120 cm). Single short or tall plants were selected from another eight lines which were segregating for height. From six segregating lines, single tall plants with height above 120 cm were selected, from one segregating line a short plant was selected with the height less than 110 cm and from another line a plant with intermediate height was chosen. The bimodal height distribution of lines is shown in Figure 5.1. Eight plants from lines with uniform height were genotyped using three

SSR markers *BARC3*, *GWM356* and *WMS4608* to identify homozygous recombinant lines. One homozygous recombinant plant from each line was harvested and together with single plants from segregating rows was progeny tested in the glasshouse. Three plants per line were grown in the glass house and measured for height at maturity. Height phenotypes for 22 short and 13 tall lines were confirmed in the glass house (Figure 5.2). Two of the four intermediate lines from the field were less than 100 cm in the glass house, thus they were classified as short, and another intermediate line was over 140 cm in height, thus classified as tall, leaving one line unclassified due to its intermediate height in both environments. Height of lines grown in the birdcage and glass house was highly correlated (Figure 5.3, $r=0.908$, $p<0.001$) with nearly all data clustering into two distinctive groups. Short lines had heights under 115 cm and tall lines were above 115 cm in both environments, except for one line (in red) which could not be confidently classified and which was excluded from the mapping family. Finally the mapping family consisted of 24 short lines carrying *Rht18* and 14 tall lines which lacked *Rht18*, and genotypes for all 38 lines were confirmed to be homozygous at the three markers.

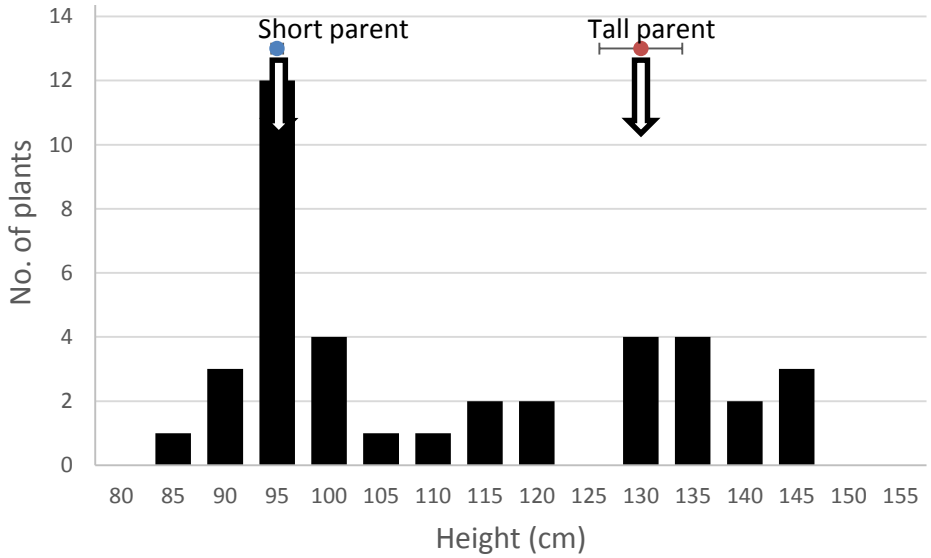


Figure 5.1 Height distributions of 39 F_4/ F_3 lines including 22 short (85-110 cm), 13 tall (130-145cm) and 4 intermediate (115-120 cm) lines together with heights of both parents (indicated as arrows with standard errors) in birdcage in 2011.

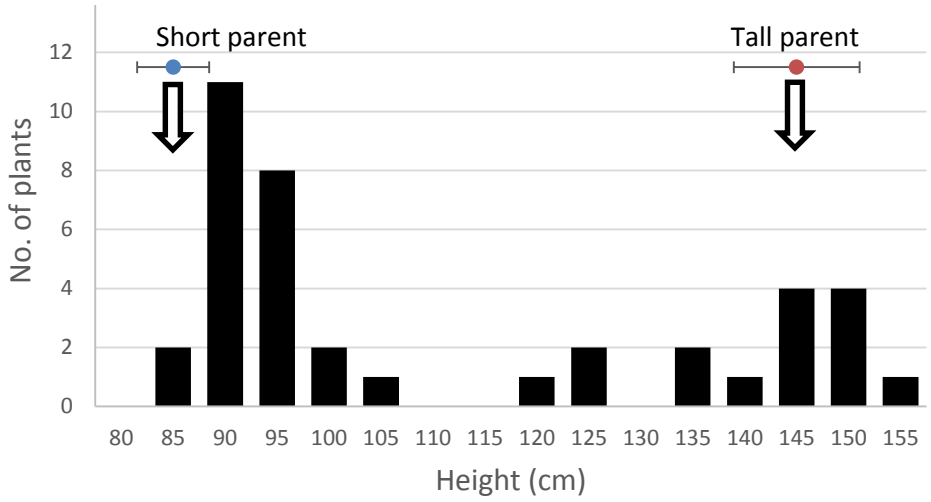


Figure 5.2 Height distributions of 39 F_5/ F_4 lines including 24 short (85-105 cm), 14 tall (125-155 cm) and 1 intermediate (120 cm) lines together with heights of both parents (indicated as arrows with standard errors) in glass house in 2012.

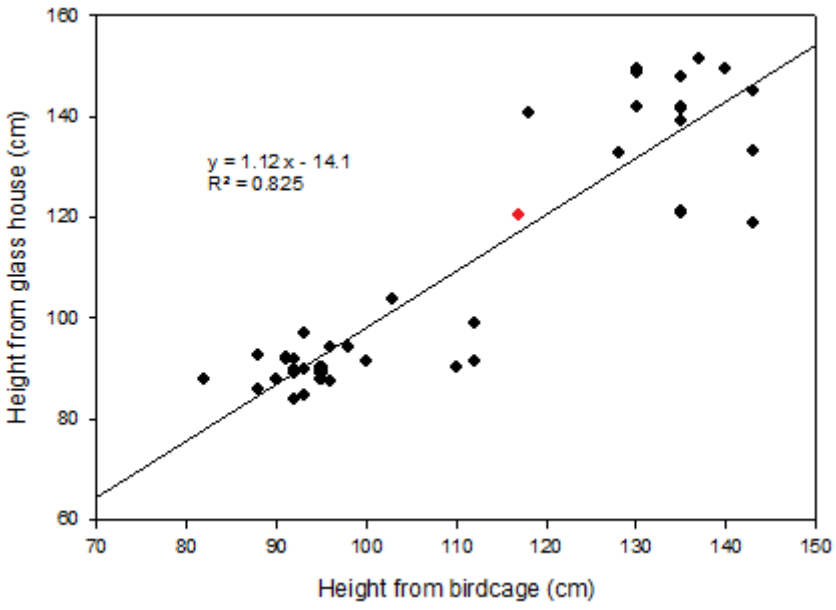


Figure 5.3 The correlation between F_4/F_3 heights from birdcage in 2011 and F_5/F_4 heights from glass house in 2012 ($p<0.001$), line with intermediate height shown in red.

5.3.2 Mapping

Initially, the mapping population of 38 lines was genotyped using three SSR markers *BARC3*, *GWM356* and *WMS4608*. The genetic distance between markers *BARC3* and *GWM356* was calculated to be 9.5 cM. There were 20 recombinants between marker *BARC3* and *Rht18* and 19 recombinants between *GWM356* and the gene (Appendix Figure 5.1), thus placing *Rht18* approx. half way between these two markers (Figure 5.4). There were no recombinants identified between SSR marker *WMS4608* and *Rht18*.

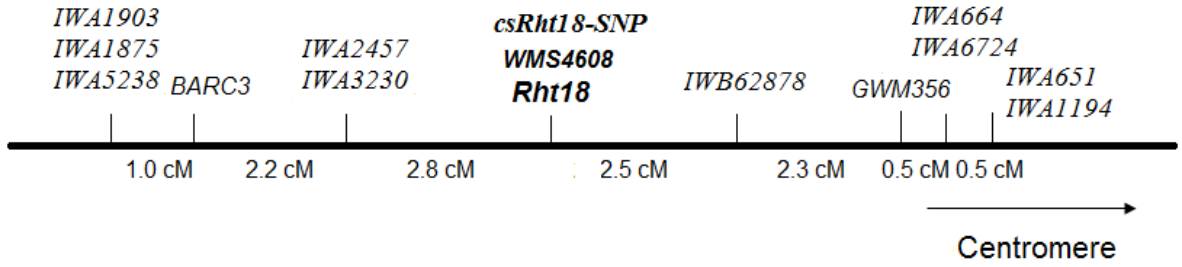


Figure 5.4 Genetic map with SNP and SSR markers linked to *Rht18* on chromosome 6AS. Unit for genetic distance is centi-Morgan (cM).

5.3.3 Identification of SNP markers

Rht18 co-segregated with SSR marker *WMS4608* on chromosome 6AS. SSR markers are not well suited for high throughput marker screening because they require complex separation steps using capillary electrophoresis when the allele difference is small. During the project a large number of SNP-based markers became available for wheat that are more amenable to high throughput technologies, therefore the next step was to screen SNP marker arrays and develop SNP-based markers linked to *Rht18*.

BSA was used to screen pooled DNAs from homozygous short and tall lines to identify linked markers using the 9K and 90K SNP array. In total, 18 short and 11 tall F_3 / F_4 lines were selected and DNAs of these lines were pooled as short and tall bulks, together with

DNAs from both parents, four bulks were analysed using 9K SNP array. Nine putatively linked SNPs were identified and converted to KASPar markers (Appendix Table 5.2). These nine KASPar markers were first confirmed to be polymorphic in parents, before they were evaluated in mapping population (Figure 5.4). Most of these SNP markers were mapped outside the previously identified genetic interval, and only two markers *IWA2457* and *IWA3230* were mapped within the interval at approx. 3.0 cM distal of *Rht18* (Appendix Figure 5.1). On the proximal side, two markers *IWA664* and *IWA6724* were the closest SNP markers which were located 0.5 cM away from SSR marker *GWM356*. No co-segregating SNP marker was identified. Later, 11 additional SNPs were selected in this region from 90K SNP array, and converted to KASPar markers. One marker *IWB62878* was mapped between *IWA664/6724* and *Rht18* on the proximal side (Figure 5.4). The new marker was the closest SNP to *Rht18* but was still separated from the gene by 2.5 cM, thus additional SNP markers were required. Previously mapped *IWA2457*, *IWA3230* and *IWB62878* were used to define the region for further marker development from the genetic SNP consensus map. Another 39 SNPs were converted to KASPar markers, but none of them showed polymorphism between short and tall parents.

5.3.4 Finding a co-segregating SNP marker

Markers which were developed from the 9K and 90K SNP arrays are not close enough to *Rht18*, and no additional marker could be generated from markers that were previously mapped to the target interval in the genetic consensus map. SSR marker *WMS4608* was still the closest marker. With the advent of physical maps and partial genome sequence of wheat, it is possible to search the region on chromosome 6A where the SSR marker is located for additional SNPs. The product of *WMS4608* was sequenced and used in blast searches of

partial genome sequence available from chromosome 6A (http://pgsb.helmholtz-muenchen.de/cgi-bin/gb2/gbrowse/Wheat_PhysMap_6A/).

An 8.2 Mbp contig (contig_6AS_1188) was identified that contained SSR marker *WMS4608* on chromosome 6A. The contig was annotated and eight genes were predicted which are listed in Table 5.1. Five predicted genes were chosen for further analysis. A fragment (4415309_6AS) which contains the SSR marker was located proximal to the five genes (Figure 5.5).

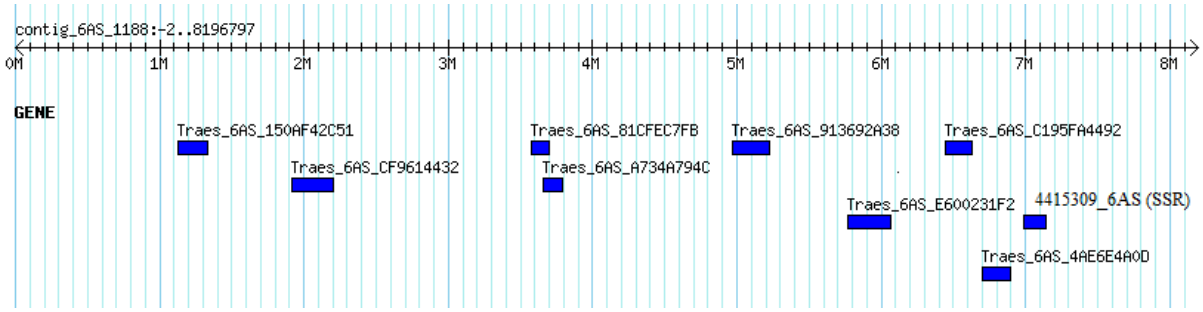


Figure 5.5 Relative positions of eight genes and the fragment 4415309_6AS which contained SSR *WMS4608* on contig_6AS_1188

Table 5.1 Annotation of eight genes on contig_6AS_1188

ID	Full name	Total size (bp)	Annotation
G1	Traes_6AS_150AF42C51*	3531	Protein phosphatise 2C
G2	Traes_6AS_CF9614432*	2350	Transcription factor E2FB
G3	Traes_6AS_81CFEC7FB	6094	Poly(A) polymerase
G4	Traes_6AS_A734A794C	1825	Poly(A) polymerase
G5	Traes_6AS_913692A38*	3480	Peroxisomal membrane protein PMP22
G6	Traes_6AS_E600231F2*	6479	Pentatricopeptide repeat-containing protein
G7	Traes_6AS_C195FA4492	2001	RNA polymerase alpha subunit
G8	Traes_6AS_4AE6E4A0D*	11710	Ubiquitin carboxyl-terminal hydrolase 15

*: Genes were chosen for further analysis for SNPs

To discover additional SNPs that may also co-segregate with *Rht18*, exons, introns and untranslated regions (UTR) of five genes (G1, G2, G5, G6, G8) were partially sequenced (Figure 5.6, Appendix Figure 5.2 and Appendix Table 5.3).

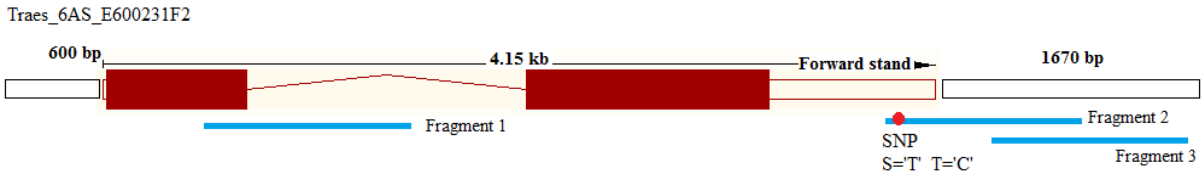


Figure 5.6 Sequenced regions of G6 on contig_6AS_1188
▬: Coding exons, White box: flanking sequence containing 5' or 3' UTR, Blue bar: Amplified 1 kb region, Red dot: identified SNP between parents, S: short parent, T: tall parent

In approx. 40 kb of DNA sequence which was generated from the contig, only two SNPs were identified. One of these SNPs was located within the contig 4415309_6AS which contained the SSR marker but could not be converted to a robust marker because it was close to a dinucleotide repeat (Appendix Figure 5.2 and Appendix Table 5.3). The second SNP was located within G6 which was predicted to encode a pentatricopeptide repeat-containing protein. The SNP was in the 3' untranslated region where a 'C' nucleotide in the tall parent was substituted for 'T' in the short parent (Figure 5.6). This SNP was converted to KASPar assay and named *csRht18-SNP*. The *csRht18-SNP* marker was then tested in the mapping family where it co-segregated with *Rht18* (Appendix Table 5.4).

5.3.5 Allele survey for *Rht18* linked markers

Molecular markers are used as surrogates for phenotypic traits such as height. Once tightly linked markers are developed, it is important to determine the frequency of the new allele in a wide range of germplasm before its utility for marker-assisted selection can be assessed. The

marker will only be useful if the frequency of the donor allele is low in backgrounds which do not carry the trait.

The SSR marker *WMS4608* and linked SNP markers were tested in 192 lines of Australian and international germplasm consisting mostly of hexaploid lines but including some tetraploid wheats. The *Rht18* associated allele of the *WMS4608* PCR product is 239 bp long, and was found in four bread wheat cultivars that were unlikely to carry *Rht18*. The 239 bp product was also found in three durum cultivars: Jandaroi, Cappelli and Castelporziano, which are not known to have Icaro in their pedigree (Table 5.2). The majority of non-*Rht18* carrying lines contained allele sizes that ranged from 219-241 bp, which required capillary electrophoresis to confidently separate product sizes.

The *Rht18* associated allele of *csRht18-SNP* was not present in bread wheats which were tested in this study. The allele was not present in the wild type durum Anhinga, but it is present in the durum cultivars: Jandaroi, Cappelli and Castelporziano, suggesting these lines carry a conserved 'Icaro-like' haplotype (Table 5.2, Figure 5.7). Other linked SNP markers showed a much higher frequency of donor allele across hexaploid and tetraploid lines, indicating that these SNPs are not as useful to select the donor allele in marker-assisted selection. *csRht18-SNP* was further tested in other bread wheat cultivars from Middle Eastern countries (74 cultivars) and from China (30 cultivars) and none of these lines contained the donor allele (data not shown). The results indicate that *csRht18-SNP* is a very useful marker in bread wheat breeding programs that are incorporating *Rht18*.

Table 5.2 Allele survey for co-segregating marker *WMS 4608*, *csRht18-SNP*, and tightly linked markers *IWA2457*, *IWA3230*, *IWB62878* in Australian wheat lines (ordered by the allele size of SSR marker *WMS4608*).

Cultivar	*WMS4608*	*csRht18-SNP*	*IWA2457*	*IWA3230*	*IWB62878*
Chinese Spring	241	B			
Anhinga*^	239	B			
Jandaroi^	239	A			
Cappelli^	239	A			
Castelporziano^	239	A			
Arrivato^	239	B			
Fortune	239	B	B	B	
Kalka^	239	B			
DBA Aurora	238	B			
Gladius	236	B			
AGT Scythe	235	B			
Axe	235	B	A	A	A
Espada	235	B	A	A	
Excalibur	235	B			
Gabo	235	B			
Kord CL Plus	235	B			
Maringa	235	B			
Merinda	235	B			
Shield	235	B			
Kunjin	234	B			
LongReach Lincoln	234	B			
Machete	234	B			
Caparoi^	233	B			
Hyperno^	233	B			
Saintly^	233	B	B	B	B
Tjilkuri^	233	B			
WID802^	233	B			
Wollaroi^	233	B			
Yallaroi^	233	B			
Yawa^	233	B			
AGT Katana	231	B			
AGT Young	231	B			
Annuello	231	B			
Aroona	231	B			
Arrino	231	B			
Baxter	231	B	B	B	B
Binnu	231	B			
To be continued					

Cultivar	*WMS4608*	*csRht18-SNP*	*IWA2457*	*IWA3230*	*IWB62878*
Bolac	231	B	B	B	B
BT-Schomburgk	231	B			
Cadoux	231	B	H	A	
Carinya	231	B			
Cascades	231	B			
Chara	231	B			
Clearfield Wht Jnz	231	B			
Cook	231	B			
Cunningham	231	B			
EGA Castle Rock	231	B			
EGA Kidman	231	B			
EGA Wentworth	231	B			
EGA Wills	231	B			
EGA Wylie	231	B			
Elmore CL PLus	231	B			
Fang	231	B			
GBA Combat	231	B			
GBA Sapphire	231	B			
Grenade CL Plus	231	B			
H46	231	B			
Impose CL Plus	231	B			
Janz	231	B			
Justica CL Plus	231	B			
Kelalac	231	B			
Krichauff	231	B			
Lang	231	B	B	B	
Livingston	231	B	B	B	A
LongReach Cobra	231	B			
LongReach Crusader	231	B			
Longreach Dakota	231	B			
LongReach Gauntlet	231	B			
Longreach Guardian	231	B			
LongReach Impala	231	B			
LongReach Spitfire	231	B			
Lorikeet	231	B			
Mackellar	231	B			
Magenta	231	B	B	B	A
Meering	231	B			
Molineux	231	B			
Naparoo	231	B			
Pelsart	231	B			
Rosella	231	B			

To be continued

Cultivar	*WMS4608*	*csRht18-SNP*	*IWA2457*	*IWA3230*	*IWB62878*
Rudd	231	B			
Sunbri	231	B			
Sunco	231	B	B	B	
Sunguard	231	B			
Sunlin	231	B			
Sunsoft 98	231	B			
Suntop	231	B			
Sunvale	231	B	B	B	
Sunvex	231	B			
Tamaroi^	231	B			
Tammin	231	B			
Tasman	231	B			
Waagan	231	B			
Wallup	231	B			
Westonia	231	B	B	B	
Whistler	231	B			
Worrakatta	231	B			
Wyalkatchem	231	B	B	B	
Giles	231	N			
Merlin	223	B			
EGA Bellaroi^	221	B			
GBA Hunter	221	B			
Kennedy	221	B	B	B	
Lincoln	221	B	A	A	
Orion	221	B			
Preston	221	B	A	A	
Tennant	221	B			
Zebu	221	B			
Zippy	221	B			
Zulu^	221	B			
Barham	219	B			
Batavia	219	B			
Bowie	219	B			
Braewood	219	B			
Brennan	219	B			
Brookton	219	B			
Bullaring	219	B			
Bumper	219	B			
Calingiri	219	B			
Carnamah	219	B			
Clearfield Wht Stl	219	B			
Cobra	219	B			

To be continued

Cultivar	*WMS4608*	*csRht18-SNP*	*IWA2457*	*IWA3230*	*IWB62878*
Corack	219	B			
Correll	219	B			
Cranbrook	219	B	B	B	
Derrimut	219	B			
Diamondbird	219	B			
Drysdale	219	B	B	B	
Dundee	219	B			
EGA Bonnie Rock	219	B			
EGA Bounty	219	B			
EGA Burke	219	B	B	B	
EGA Eagle Rock	219	B			
EGA Eaglehawk	219	B			
EGA Gregory	219	B	B	B	
EGA Hume	219	B			
EGA Jitarning	219	B			
EGA Stampede	219	B			
EGA Wedgetail	219	B			
Einstein	219	B			
Ellison	219	B			
Emu Rock	219	B			
Endure	219	B			
Estoc	219	B	B	B	A
Festiguay	219	B			
Forrest	219	B			
Frame	219	B			
GBA Ruby	219	B			
Glover	219	B			
H45	219	B			
Halberd	219	B	B	B	
Hartog	219	B	B	B	
Impala	219	B			
King Rock	219	B			
Kukri	219	B			
Lancer	219	B			
Longreach Beaufort	219	B			
Longreach Catalina	219	B			
LongReach Dart	219	B			
LongReach Envoy	219	B			
LongReach Gazelle	219	B			
LongReach Orion	219	B			
LongReach Phantom	219	B			
LongReach Scout	219	B			

To be continued

Cultivar	*WMS4608*	*csRht18-SNP*	*IWA2457*	*IWA3230*	*IWB62878*
Mace	219	B	B	B	A
Mansfield	219	B			
Marombi	219	B			
Peake	219	B			
Petrel	219	B			
Pugsley	219	B			
QAL2000	219	B			
QALBIS	219	B			
Raven	219	B			
Rees	219	B			
Scout	219	B	B	B	A
Sentinel	219	B			
Seri 82	219	B			
Silverstar	219	B	B	B	
Spear	219	B			
Spitfire	219	B	B	B	A
SQP Revenue	219	B			
Stiletto	219	B			
Strzelecki	219	B			
Sunelg	219	B			
Sunstate	219	B	B	B	
Sunzell	219	B			
Tammarin Rock	219	B			
Thatcher	219	B			
Trident	219	B			
Ventura	219	B			
Wedin	219	B			
Wylah	219	B			
Yandanooka	219	B			
Yenda	219	B			
Yitpi	219	N	B	B	
Harrismith		B			

*Anhinga used as control, ^: tetraploid wheat, Short parent (donor) = 239bp =A, Tall parent= 233bp = B in SSR and SNP marker

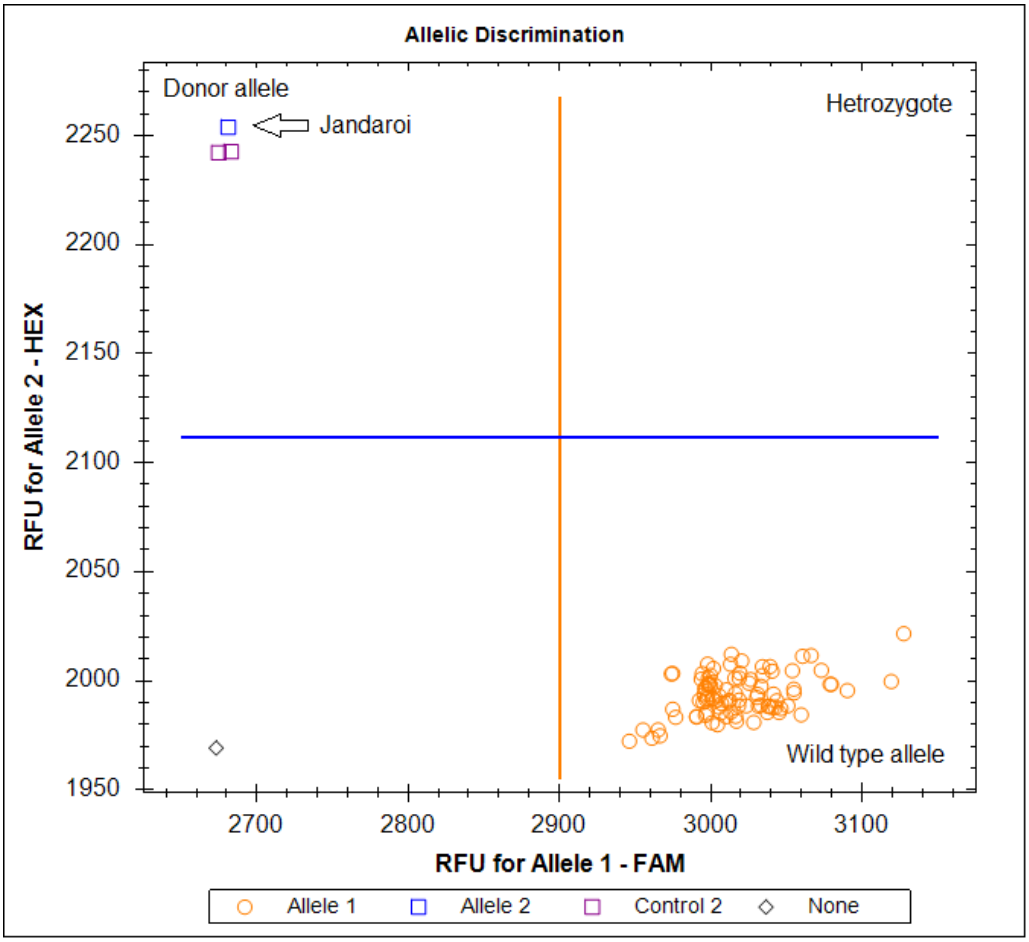


Figure 5.7 Allelic discrimination of SNP marker *csRht18-SNP* tested on part of Australian wheat validation panel using the KASPar assay. RFU: relative fluorescence unit, Allele 1: wild type allele, Allele 2: donor allele, Control 2: Icaro.

5.4 Discussion

This study focused on fine mapping an alternative semi-dwarfing gene *Rht18* and developing a co-segregating SNP marker to assist future breeding programs aiming to replace *Rht-B1b/D1b* with *Rht18*.

Reliable height phenotyping of a segregating family is essential before *Rht18* can be mapped accurately. In this study F_2 lines derived from the cross between Langdon and Icaro were progeny tested in both field and glass house to confirm the true phenotype and lines which could not be confirmed in both environments were discarded. Using marker assisted selection and inbreeding, a mapping family was developed that consisted of only homozygous

lines that were fixed for the presence or absence of *Rht18*. This approach allowed the accurate positioning of *Rht18* between flanking markers *BARC3* and *GWM356* on chromosome 6AS. In previous genetic mapping study of *Rht18* by Haque et al. (2011), only F_2 lines from Icaro × Langdon were phenotyped once without further progeny tests. Lack of robust phenotyping probably explained the discrepancy in the genetic maps between this study and previously published map in the order of the common marker *BARC3* and *Rht18* (see Appendix Figure 5.3).

To accurately determine the size of SSR markers, PCR products usually have to be separated by capillary electrophoresis while SNP genotyping only requires the quantification of the abundance of PCR products by measuring the relative fluorescence levels associated with each allele. SNP genotyping is therefore more amenable to high throughput screening technologies which are favoured by plant breeders today. This was the main reason why a large amount of effort was directed towards the identification of a co-segregating SNP-based marker, although the SSR marker *WMS4608* was already available. Once a SNP marker was identified from the SNP array, the marker was converted into ready-to-use format such as the KASPar assay.

Finding polymorphisms between mapping parents is a key step to develop a tightly linked marker. In this study three different approaches were used.

Firstly, BSA was used to find SNPs linked to *Rht18*. BSA is a quick way to identify linked markers located in specific chromosomal regions based on contrasting DNA bulks derived from lines with different phenotypic expression of a trait (Michelmore et al. 1991). Both 9K and 90K SNP arrays were screened with DNA extracted from parental lines and bulks which contained randomised loci except for the region containing *Rht18*. This approach yielded several tightly linked markers, but no co-segregating marker.

Secondly, most of the SNP markers on the arrays have previously been mapped using several individual mapping populations. A consensus map that combined markers from

individual maps and mapping populations, can be screened for additional markers in the target region. Although all markers on the array in theory would have been assayed during the BSA, the screening process relies on small changes in DNA hybridisation between samples which may have missed some polymorphic markers. To test this hypothesis, it was decided to convert several markers within the *Rht18* interval into KASPar assays and test for polymorphism between parental lines. None of the 39 SNPs from the *Rht18* interval which were converted into KASPar assays identified polymorphism between parental lines. Using this approach no additional marker was mapped in the target region.

Finally, the partial genome sequence which was generated in the cultivar Chinese Spring as part of the International Wheat Genome Sequencing Consortium was exploited for marker development. The co-segregating SSR marker *WMS4608* was used to identify a contig of approx. 8Mb of genomic sequence that contained the SSR marker. This contig contained several predicted genes which were sequenced in parental lines. One SNP was identified between parental lines, converted into KASPar assay and mapped genetically to the *Rht18* locus. The marker *csRht18-SNP* proved a very useful marker because the *Rht18* associated allele was absent in hexaploid wheat. The marker is expected to be informative in a wide range of hexaploid germplasm and will assist in the selection for *Rht18* in breeding programs where other dwarfing genes may also be present.

Unexpectedly, the Icaro allele of *csRht18-SNP* was absent in Anhinga, although Icaro was derived from Anhinga and was predicted to carry the same allele. It is also possible that the Anhinga used in this study is not the genuine Anhinga which was used to generate Icaro after mutagenesis. However, four Anhinga lines with different accession numbers (AUS15091, AUS17232, AUS16025 and AUS24454) were tested with *csRht18-SNP*, all showing the tall parent genotype. It is not known if these accessions included the line which was used in the mutagenesis experiment. Additionally, spontaneous mutation cannot be ruled out, as it always occurring and the substitution of C by T is a common event. It is possible

that the SNP was caused by the random mutagenesis treatment in Icaro. However the presence of the same allele in other durum germplasm such as Jandaroi makes this unlikely since Icaro is not in the pedigree of Jandaroi. It is also unlikely that the SNP itself is responsible for the dwarfing phenotype given that the single base pair polymorphism is not characteristic for mutation generated by fast neutron radiation and it was located outside the open reading frame of a predicted gene. The identification of a candidate gene for *Rht18* will shed light on how radiation treatment which is predicted to cause small deletions resulted in a dominant, reduced height phenotype.

5.5 Conclusion

Rht18 was mapped to chromosome 6AS in durum wheat and co-segregated with the SSR marker *WMS4608*. Screening the newly developed SNP array for wheat yielded linked markers but none of them co-segregated with *Rht18*. The emerging genome sequencing on chromosome 6A was utilised to identify SNPs in annotated genes. One of these SNPs co-segregated with *Rht18* and was converted into a robust KASPar assay. Because the SNP variant that was associated with *Rht18* was not found in bread wheat cultivars, it is predicted that the marker will play an important role in breeding programs that introduce *Rht18* as alternative to *Rht-B1b/D1b*. Because the marker is based on a single nucleotide polymorphism, it will facilitate the application in high throughput marker platforms which are commonly in place in breeding programs.

Chapter 6 Relationship of *Rht18* to other induced dwarfing genes *Rht14* and *Rht16* in durum wheat

6.1 Introduction

Previous chapters reported the detailed characterisation of *Rht18* which was originally identified in durum wheat. Tall durum Anhinga was treated with fast neutron radiation and a semi-dwarf line was isolated which was later released as cultivar Icaro. Apart from *Rht18*, other induced dwarf mutants were previously isolated in durum wheat including *Rht14* and *Rht16* which showed agronomic potential (Bozzini 1974; Konzak 1987). In this chapter, *Rht14* and *Rht16* will be evaluated for effects on coleoptile length, and their genetic relationship to *Rht18* will be determined.

The *Rht14* dwarfing gene was identified after mutagenesis of the Italian cultivar Cappelli which generated the semi-dwarf line Castelporziano (Bozzini and Scarascia-Mugnozza 1967). Mutagenesis was carried out by the same institute (the Casaccia Nuclear Research Center, CNEN, Roma) in Italy and using similar mutagen (thermal neutrons, fast neutrons for *Rht18*) which was used to identify *Rht18*. Similar to *Rht18*, *Rht14* was found to be dominant meaning that the F_1 heterozygous plant was short (Bozzini and Scarascia-Mugnozza 1967). The Castelporziano semi-dwarfing gene was later transferred into several commercially successful cultivars (Giorgi et al. 1984). Compared with Cappelli, *Rht14* reduced height by approx. 30% and it provided significant lodging resistance (Scarascia-Mugnozza et al. 1993). A similar height reduction of approximately 29% was observed for *Rht18* (Chapter 5).

Another reduced height mutant was identified in durum cultivar Edmore by Konzak in United States of America (Konzak 1987). Mutant Edmore M1, also known as Edmore MUTSD1 (PI 499362), was identified after treating durum wheat cultivar Edmore (CI17748)

with the chemical mutagen ethyl methanesulfonate (EMS) (Maluszynski and Szarejko 2003). The height reducing gene in Edmore M1 was found to be partially dominant and designated as *Rht16* (Konzak 1988). The gene was reported to have good breeding potential (Konzak 1988a). In a later study, *Rht16* in Edmore M1 reduced height by approx. 25% compared with Edmore which is less severe than what was reported for *Rht14* and *Rht18* (Ellis et al. 2004).

Castelporziano and Icaro were developed from different cultivars using same physical mutagenesis in Italy, while Edmore M1 was generated from Edmore, an American cultivar, by chemical mutagenesis. Possible genetic relationships between *Rht14*, *Rht16* and *Rht18* were reported by Haque et al. (2011) where *Rht18* was found to be allelic to *Rht14* and *Rht16* and mapped to the short arm of chromosome 6A. However, the classification of short and tall phenotypes in three mapping families for *Rht14*, *Rht16* and *Rht18* derived from Castelporziano, Edmore M1, Icaro crossed to Langdon were ambiguous. For instance, the classified tall lines ranged from 119 to 154 cm in the Castelporziano by Langdon cross, while the same class ranged from 135 to 154 cm in the Edmore M1 by Langdon cross. Given Castelproziano and Edmore M1 had similar heights (73 cm and 77 cm), progeny testing of F_2 populations would have been necessary to confirm phenotypes before drawing any conclusions about possible allelism.

In Chapter 3, *Rht18* was reported not to reduce coleoptile length in durum and hexaploid wheat. It was therefore important to also characterise *Rht14* and *Rht16* for possible effects on coleoptile length. Previously, Castelporziano and Edmore M1 were reported to have long coleoptiles but no data was provided to back up this claim (Konzak 1987; Konzak 1988). When coleoptile lengths of Castelporziano and Edmore M1 were compared to tall counterparts (Ellis et al. 2004), mutants had shorter coleoptiles than wild types. These results may have been confounded by the effect of background mutations on general growth and vigour of mutants when compared to wild type as suggested for *Rht18* in Chapter 3.

The aim of this study was to determine if *Rht18* is allelic to *Rht14* or *Rht16* by intercrossing Icaro with Castelporziano and Edmore M1. If tall plants were observed in F_2 progeny derived from these crosses, the dwarfing gene can be determined as non-allelic to *Rht18* and the genetic location of the gene needs to be investigated. Mapping families were also scored for coleoptile length to assess possible effect of *Rht14* and *Rht16* on this trait.

6.2 Materials and methods

6.2.1 Populations

6.2.1.1 Populations for allelism tests

Three crosses were made between the three semi-dwarf parents: Icaro × Castelporziano (*Rht18* × *Rht14*) (Expt 13, Table 6.1), Icaro × Edmore M1 (*Rht18* × *Rht16*) (Expt 14) and Castelporziano × Edmore M1 (*Rht14* × *Rht16*) (Expt 15). For Expt 13 and Expt 14, 183 F_2 plants from each cross were evaluated for height outside in the birdcage in 2013. 169 F_2s of Expt 15 were assessed for height in the glass house in April 2014.

6.2.1.2 A mapping population that was segregating for *Rht16*

To eliminate *Rht18*, 183 F_2 plants generated from Expt 14 were screened with SSR marker *WMS4608* which was previously shown to be tightly linked to *Rht18* and polymorphic in parents. A subset of 82 lines that lacked *Rht18*-associated alleles were advanced to F_3 by single seed descent (SSD). F_3 families (16 plants per line) were progeny tested in the glasshouse for height, and four plants were harvested from each family. F_4 families were progeny tested in rows in birdcage in July 2014. The *Rht16* mapping family consisted of 26 homozygous short and 34 homozygous tall lines and a subset of these lines was used for selective genotyping analysis (see 6.2.3).

6.2.1.3 Population for coleoptile assessment

Durum cultivar Capelli (AUS10389, *Rht14* wild type) was crossed to short mutant line Castelporziano (AUS15950) carrying *Rht14* to generate F_1 (Expt 17) which was then selfed to generate F_2 population for coleoptile length assessment. Similarly, the F_1 (Expt 18) and F_2 generation (Expt 20) were obtained by crossing tall Edmore (AUS 19781, *Rht16* wild type) to short Edmore M1 (*Rht16* mutant) (AUS25288). The heights of F_1s were measured together with both parents to assess the gene action of *Rht14* and *Rht16*. Approx. 120 F_2 seeds from both population Expt 19 and Expt 20 were sown in trays to conduct coleoptile length assessment.

Table 6.1 Populations deployed in allelism survey with sowing dates (Abbreviation: Pop, population; Cast, Castelporziano; EdM, Edmore M1; Dom, dominance; Col Asse, coleoptile assessment).

ID	Pop	Parent 1	Parent 2	Allelism test	Mapping	Dom test	Col Asse
Expt 13	F_2	Icaro (*Rht18*)	Cast (*Rht14*)	Sep 2013			
Expt 14	F_2	Icaro (*Rht18*)	EdM (*Rht16*)	Sep 2013			
Expt 15	F_2	Cast (*Rht14*)	EdM (*Rht16*)	Apr 2014			
Expt 16	F_4	Icaro (*Rht18*)	EdM (*Rht16*)		Jul 2014		
Expt 17	F_1	Cast (*Rht14*)	Capelli			√	
Expt 18	F_1	EdM (*Rht16*)	Edmore			√	
Expt 19	F_2	Cast (*Rht14*)	Capelli				√
Expt 20	F_2	EdM (*Rht16*)	Edmore				√

6.2.2 Phenotyping

Plant height was measured from the soil surface to the tip of the spike excluding awns and recorded in centimetres (cm). Height of progeny was compared to short or tall parents in glasshouse and birdcage experiments.

6.2.3 Genotyping and marker development

6.2.3.1 Genotyping for *Rht18*

Leaf tissue was harvested and DNA was extracted individually for each line from populations in Expt 14 and Expt 15. Two SNP markers *IWA2457, IWB62878* (flanking *Rht18*) and one SSR marker *WMS4608* (co-segregating with *Rht18*) were tested in the above populations to predict the presence/absence of *Rht18* (Protocols were included in Chapter 5). *WMS4608* was further tested in F_4 individuals from Expt 16 to confirm that the progeny lacked *Rht18*.

6.2.3.2 Mapping of *Rht16*

The 60 F_4 lines from the *Rht16* mapping family (Exp16) were classified for height by growing lines as rows in the birdcage. DNA was extracted from several plants within each row for subsequent mapping. Eight homozygous short and eight homozygous tall lines together with parental lines were selectively genotyped using the 90K SNP array platform. (Agriculture, Energy & Resources, Department of Economic Development, Jobs, Transport and Resources, Victoria).

Putatively linked SNP makers were converted to KASP assays (protocol for KASP primer design and assay development outlined in Chapter 5). The new KASP markers were first tested for polymorphism in parental lines, and then in mapping population.

6.2.4 Gene action and coleoptile length assessment for *Rht14* and *Rht16*

In population Expt 17 and Expt 18, the heights of F_1 plants and parental lines were measured in pots in the glasshouse to determine the dominance of *Rht14* and *Rht16*. Coleoptile lengths of F_2 plants in Expt 20 were scored (methodology outlined in Chapter 3), and then the F_2s were transplanted to grow to maturity. The final F_2 plant heights were measured to define short and tall phenotypic classes which were used for coleoptile length analysis. The coleoptile length and plant height of the chosen F_2 lines and short and tall parents were analysed for genotypic differences by Genstat 16th edition.

6.3 Results

6.3.1 Gene action of *Rht18, Rht14* and *Rht16* in durum wheat

The dominance of recessiveness of gene action of three semi-dwarfing genes *Rht14*, *Rht16* and *Rht18* waw examined by comparing final heights of F_1 heterozygotes with tall and short parental lines. Averaged height for *Rht14* F_1 heterozygote was not different from the short parent Castelporziano (Table 6.2), but different from the tall parent Cappelli, suggesting that *Rht14* is dominant in tetraploid wheat. Similarly, *Rht18* F_1 heterozygotes were the same height as short parent Icaro but different to tall Anhinga (data not shown). Averaged height for *Rht16* F_1 heterozygotes was intermediate between the parents ($P<0.001$), suggesting this gene is semi-dominant in tetraploid background.

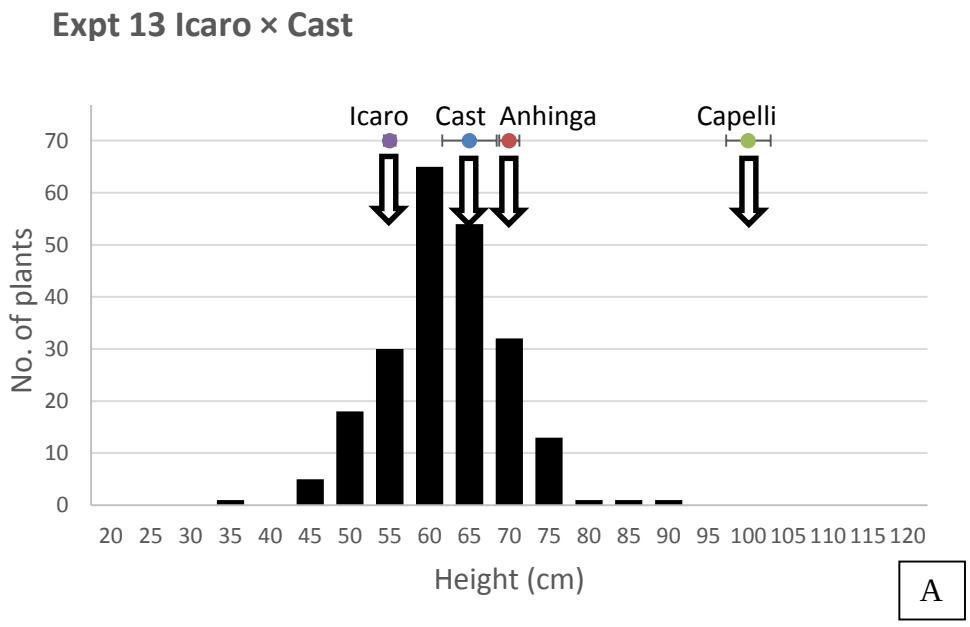
Table 6.2 Averaged heights of parents and F_1 lines from population Expt 17 and Expt 18 (The heights of short and tall parents were compared to F_1 in each population to determine P value using T-test).

Population	Line	No. of plants	Mean (cm)	P value
Expt 17	Castelporziano (*Rht14*)	4	65.2	ns
	Capelli	7	102	***
	F_1	14	70.6	
Expt 18	Edmore M1 (*Rht16*)	4	71.2	***
	Edmore	4	101	***
	F_1	21	85.5	

***: $P<0.001$, ns: not significant

6.3.2 Height distributions and allelism tests

Three mutant lines carrying *Rht14*, *Rht16* and *Rht18* were crossed to each other to generate three F_2 populations. Height distribution of each F_2 population was compared to their corresponding parents and possible allelic relationships of three semi-dwarfing genes were examined.



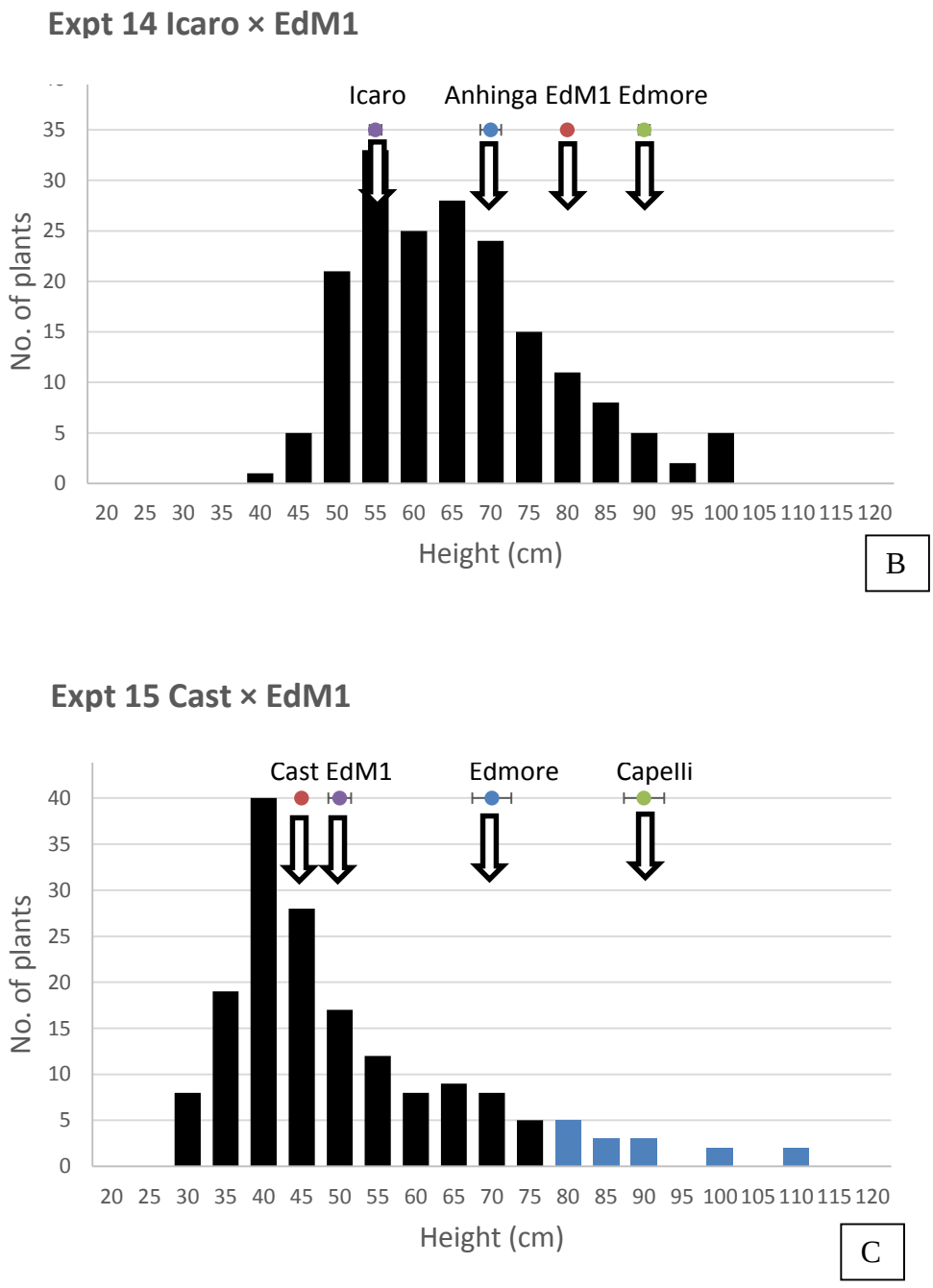


Figure 6.1 Height distributions of F_2s from crosses between Icaro (*Rht18*), Castelporziano (*Rht14*) and Edmore M1 (*Rht16*). Heights of mutant and wild type parents were indicated in each population with error bars showing standard errors. 15 lines in blue in Expt 15 were genotyped in Section 6.3.3 (Abbreviation: Cast, Castelporziano; EdM1, Edmore M1).

The height distributions of F_2 plants from each cross were shown in Figure 6.1. The height distribution is symmetric in population Expt 13 (*Rht14* × *Rht18*) with above 95% F_2s

within the range between 40 cm and 80 cm which is close to both short parents. The tall control Capelli was taller than the tallest F_2 plant and measured approx. 100cm. There were 16 plants taller than Anhinga but shorter than Capelli in this population. It is possible that these lines carry none of the dwarfing genes, but given the symmetrical normal distribution of heights, it is likely that these tall lines are just outliers contributed by background genes from Capelli, thus not true tall segregants. Further progeny tests will confirm this result. However, the height distribution graph indicates that *Rht18* and *Rht14* are likely to be allelic to each other.

In population Expt 14 (*Rht16* × *Rht18*), the height distribution is asymmetric and skewed towards the tall class. Most of the lines fall within the range between 45 cm and 85 cm, however, there were a number of F_2 plants as tall as or taller than Edmore (91cm) classified as tall lines. The result suggested that *Rht16* and *Rht18* are linked or on different chromosomes thus, they are not allelic. This population was progeny tested to generate a mapping family for *Rht16* (see Section 6.3.4).

The height distribution in Expt 15 (*Rht14* × *Rht16*) had a similar shape to that in Expt 14. The majority of plants measured between 30 cm and 75 cm, but there were 15 plants taller than 80 cm. There were four plants taller than 100 cm, which indicated that *Rht14* and *Rht16* were unlikely to represent mutations within the same gene.

In summary, height measurements from progeny of intercrosses suggested that *Rht18* is likely to be allelic to *Rht14* but *Rht16* is probably in a location different to the other two genes.

6.3.3 Chromosome location of *Rht14*

If *Rht14* is allelic to *Rht18*, both genes should be located on 6AS and markers linked to *Rht18* should also map close to *Rht14*. Therefore the tallest plants from Expt 15 (*Rht14* × *Rht16*)

should carry the non-*Rht14* associated allele of SSR marker *WMS4608*. If confirmed, this would indicate that *Rht14* is located on 6AS as is *Rht18*. Expt 15 with 169 F_2 plants (Figure 6.1C) was derived from the cross between the Castelporziano and Edmore M1. The *Rht18* linked markers, including two SNP markers (*IWB62878* and *IWA2457*) and the co-segregating SSR marker *WMS4608* (Chapter 5), were polymorphic between the parents allowing these markers to be used in this population. The 15 tallest plants (height ranged from 76 to 110 cm) were chosen for marker analysis and genotyped using three *Rht18* linked markers (Table 6.3). Co-segregating marker *WMS4608* showed complete association of non-*Rht14* associated allele with tall phenotype, indicating that *Rht14* is linked to this marker and therefore located on 6AS. Other SNPs markers were also linked but not perfectly, probably due to recombination between marker and the gene. The result is consistent with previous results, suggesting that *Rht14* and *Rht18* are alleles of a gene located on chromosome 6AS.

Table 6.3 Genotypes of height ranked F_2 lines from Castelporziano × Edmore M1 in Expt 15

Line ID	Height (cm)	*IWB62878*	*WMS4608*	*IWA2457*
1	76	B	B	B
2	78	B	B	B
3	79	B	B	B
4	80	B	B	B
5	80	B	B	B
6	81	B	B	H
7	82	B	B	H
8	85	B	B	B
9	86	B	B	H
10	88	B	B	B
11	90	H	B	B
12	96	B	B	B
13	100	B	B	B
14	107	B	B	H
15	110	B	B	H
Castelporziano	45	A	A	A
Edmore M1	56	B	B	B
Cappelli	88	A	A	A
Edmore	78	B	B	B

6.3.4 Mapping of *Rht16*

Rht16 was proposed to be non-allelic to *Rht18* and *Rht14*. *Rht18* and *Rht14* are on Chromosome 6A according this study, but the chromosome location for *Rht16* remains unknown. To map *Rht16*, 141 F_2s from Expt 14 which contained the *Rht18* associated allele were eliminated from 183 lines using the co-segregating marker *WMS4608*, leaving 42 F_2s segregating only for *Rht16* (Figure 6.2). The population was expanded by growing a further 200 F_2 lines in trays in glass house and 40 lines were selected as homozygous for non-*Rht18* allele using the same marker. Altogether 82 F_2 lines free from *Rht18* allele were progeny tested in glass house to generate F_3 and in birdcage for F_4 lines. Among these 26 short and 34 tall F_4 lines were confirmed to be homozygous in Expt 16. Height distribution of 60 F_4 lines showed the clear separation of two groups judged by the short and tall controls (Figure 6.3).

Selective genotyping provided linked SNPs on 90K array for *Rht16* and suggested that this gene is most likely located on chromosome 5B since the linked SNPs named as *IWB33231*, *IWB42727*, *IWA6837*, *IWB53020*, and *IWB69519* were located on chromosome 5B (Appendix Table 6.1). The five SNPs were converted into KASP assays (Appendix Table 6.2) and tested for polymorphism in Icaro and Edmore M1. Primers from *IWB42727* and *IWB69519* showed polymorphism between the parents, and the two markers were then further genotyped on 60 F_4 lines in population Expt 16. Inconsistencies of genotype with phenotype were found at both markers (Table 6.4), suggesting that neither marker is co-segregating with *Rht16*. However, if both markers were unlinked, we would expect to see approx. 30 lines which were inconsistent between genotype and phenotype but linkage analysis only identified 13 lines where genotype was not matching phenotype. Chi square test for *IWB42727* (χ^2=11.2, P<0.01) and *IWB69519* (χ^2=12.2, P<0.01) indicated that the hypothesis of 1:1 segregation ratio can be rejected for both markers. We conclude both markers are linked with *Rht16* on Chromosome 5B.

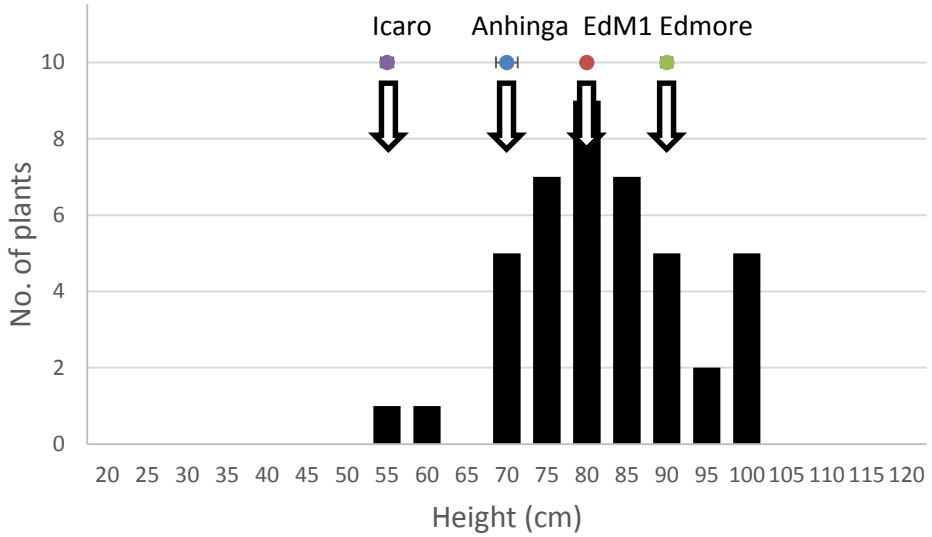


Figure 6.2 Height distributions of 42 F_2 lines homozygous for non-*Rht18* associated allele. Heights of mutant and wild type parents were indicated with error bars showing standard errors.

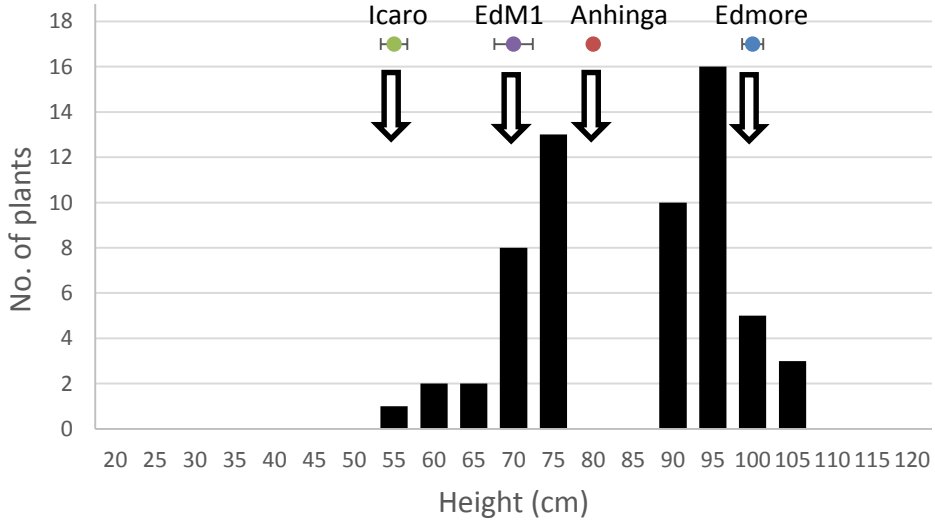


Figure 6.3 Height distributions of 60 F_4s of Expt16 derived from Icaro and Edmore M1. Heights of mutant and wild type parents were indicated with error bars showing standard errors.

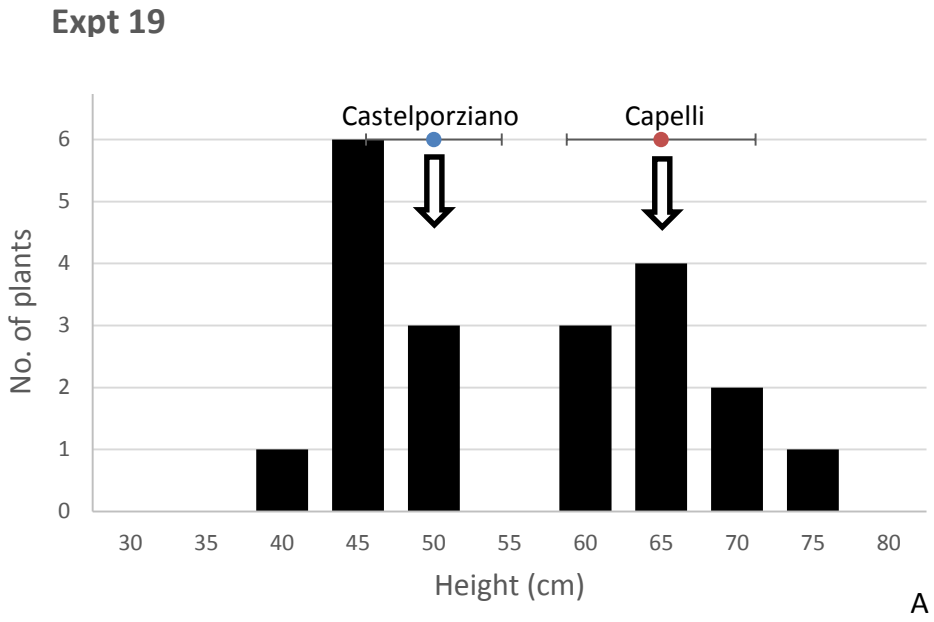
Table 6.4 Number of lines found with an inconsistent genotype to phenotype by two SNP markers in different height class in Expt 16 (Lines with heterozygous or an opposite genotype to phenotype were recorded as mismatch).

Height class	*IWB42727*		*IWB69519*	
	mismatch	match	mismatch	match
Short	7	19	6	20
Tall	5	29	5	29
Total	12	48	11	49

6.3.5 Do *Rht14* and *Rht16* affect coleoptile length in durum wheat?

Parental lines Castelporziano and Capelli were different in height in Expt 19 (Appendix Table 6.3). Approx. 40 F_2 lines were tested for height and coleoptile length, among them 10 lines were equivalent or shorter than Castelporziano (51 cm), and were thus selected as short lines for coleoptile assessment. Another 10 lines taller than 60 cm, similar or taller than Capelli (65cm) were selected as tall. The population consisted of 20 lines, and had a bimodal segregation for height (Figure 6.4 A). In Expt 20, Edmore M1 and Edmore were different in height (Appendix Table 6.3). Approx. 100 F_2 lines were tested for height and coleoptile length, among them 24 lines were equivalent or shorter than 40 cm and selected as short. Another 25 lines were taller than 50 cm (averaged Edmore = 47 cm), thus selected as tall. The height distribution showed a bimodal pattern (Figure 6.4 B). Since *Rht16* is a semi-dominant gene, heterozygote lines should have an intermediate height, and those selected lines were considered as homozygous in Expt 20. Coleoptile length was reduced in both Castelporziano and Edmore M1 mutant parents. However, when selected short and tall F_2 lines were compared there was no difference in both populations (Figure 6.5), suggesting that *Rht14* and *Rht16* have no detrimental effect on coleoptile length. In both populations, coleoptile lengths of short F_2 lines were longer than Castelporziano or Edmore M1 and tall F_2 lines had shorter

coleoptile length than Capelli or Edmore, consistent with a reduction in the strength of 'background' mutations as a result of crossing and segregation. A wide range in coleoptile length was found in both short and tall progenies including outliers, presumably due to the segregation of minor genes. These results are consistent with the results of *Rht18* in tetraploid wheats, suggesting three semi-dwarfing genes are unlikely to reduce coleoptile length (Chapter 3).



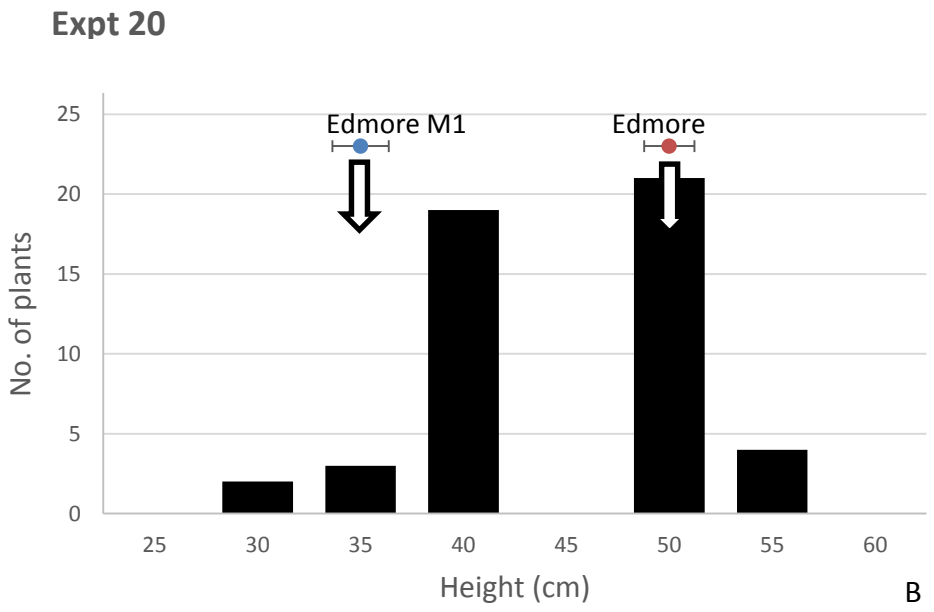


Figure 6.4 Height distributions of Expt 19 derived from Castelporziano × Capelli and Expt 20 derived from Edmore M1 × Edmore. Heights of mutant and wild type parents were indicated in each population with error bars showing standard errors.

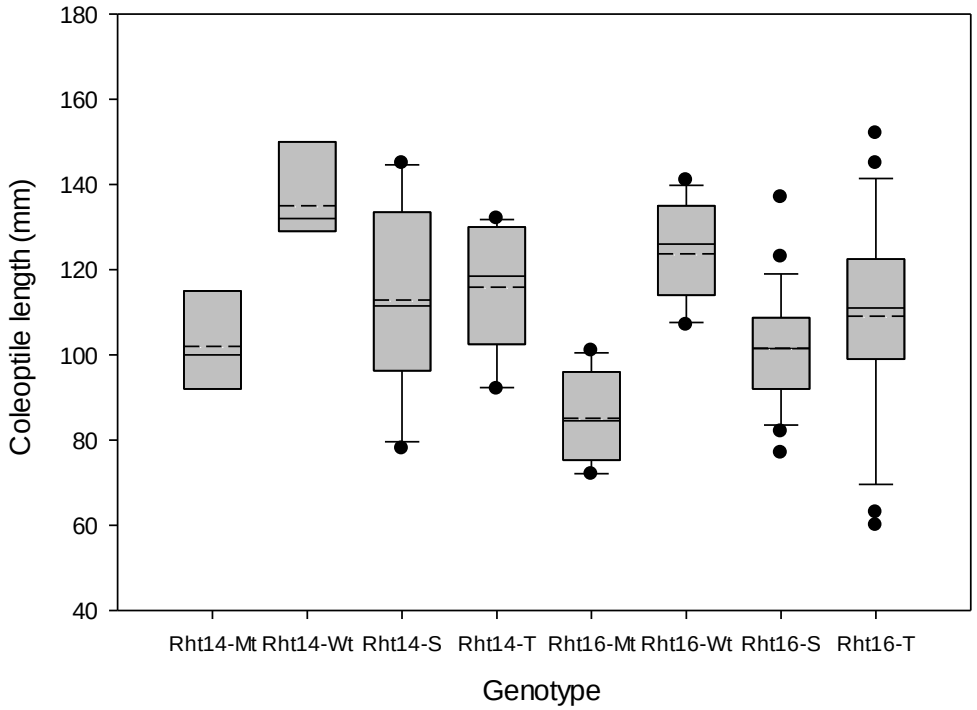


Figure 6.5 Coleoptile length assessments for mutant and wild type, and short and tall F_2 lines from populations segregating for *Rht16* and *Rht14*. From left to right, *Rht14* mutant, *Rht14* wild type, *Rht14* short F_2s, *Rht14* tall F_2s, *Rht16* mutant, *Rht16* wild type, *Rht16* short and *Rht16* tall F_2s. The lower and upper edges of the box represent 25th and 75th percentiles, and the solid and dashed lines are the medians and means in each box. The 'error bars' indicates 10th and 90th percentiles; while the filled circles are outliers in each class.

6.4 Discussion

The dwarfing effect of *Rht14* was shown to be dominant and *Rht16* was classified as a semi-dominant gene, which is consistent with previous studies (Bozzini and Scarascia-Mugnozza 1967; Konzak 1988). *Rht18* was also classified as a dominant gene (Section 6.3.1) in contrast to the initial report which categorised *Rht18* as a semi-dominant gene (Konzak 1987).

Correct phenotyping is critical to determine the results of allelism tests, and it relies on both tall and short controls in each experiment to identify any 'Tall' segregants. In Icaro × Catelporziano (Expt 13), the F_2 population had a symmetrical normal distribution, and no plants were recovered which were as tall as the wild type control Capelli. There were several lines which were taller than the tall parent Anhinga (Figure 6.1), but given the symmetrical

height distribution, it is likely that these lines are probably not true tall segregants, although this would need to be confirmed through progeny testing. Based on these results, it is likely that *Rht14* and *Rht18* are either alleles or mutations within closely linked genes. In Icaro × Edmore M1 (Expt 14), some tall plants were clearly taller than both wild type controls Edmore and Anhinga and the height distribution was skewed towards the tall category indicating that *Rht18* and *Rht16* are probably independent loci because tall segregants were recovered. In Castelporziano × Edmore M1 (Expt 15), again some plants were taller than both wild type controls Edmore and Cappelli, and therefore classified as tall lines. Crossing *Rht14* with *Rht16* in Expt 15 was a supplemental test confirming conclusions from results obtained in Expt 13 and Expt 14. In conclusion, results from Expt 13, 14 and 15 provided evidence for allelism or linkage of *Rht18* and *Rht14* and indicated that *Rht16* is probably independent. These results contradict Haque et al. (2011) who reported that *Rht16* was allelic to *Rht14* and *Rht18*. Because no height distributions of controls were published by Haque et al, it was difficult to evaluate results. It is therefore possible that tall segregants were recovered but not recognised as such by these authors.

Both *Rht14* and *Rht18* are dominant dwarfing genes, and they reduce plant height by about 30% relative to wild type controls. Molecular markers linked to *Rht18* were also linked to *Rht14*, indicating that genes are either the same or closely linked on the same chromosome. Both mutants were generated through physical mutagenesis experiments carried out in the same institute in Italy. It is possible that the random mutagenesis induced mutations in the same gene that were responsible for dwarf phenotype in different backgrounds. The identification of the underlying mutations associated with *Rht14* and *Rht18* are required to confirm this hypothesis.

If *Rht14* and *Rht18* are allelic, they should behave very similar with respect to coleoptile length. According to results reported in Chapter 3, *Rht18* had no effect on coleoptile length in durum background. The F_2 population derived from Castelporziano ×

Capelli cross was bimodally distributed for height which was subsequently screened for coleoptile length. Although approximately 2/3 of the short plants were predicted to be heterozygous, a lack of correlation between height and coleoptile length indicated that *Rht14* did not reduce coleoptile length. For *Rht16,* due to the semi-dominance of this gene, F_2 lines were selected from tails (shortest and tallest) of the population which were predicted to be homozygous. Ideally, progeny test is required to confirm the result. Again, lack of correlation between height and coleoptile length was observed in this population. Differences in coleoptile length observed between mutants were not observed in the progenies of *Rht14* and *Rht16* crosses, similar to the results obtained from the *Rht18* coleoptile screen, and the reasons for this were discussed in (Section 3.4.1, Chapter 3). Therefore, it is important to compare coleoptile length in a segregating population rather than just between mutant and wild type.

Rht16 was mapped to chromosome 5B using linked markers that were identified from screening the 90K SNP array with short and tall lines. This result is consistent with the conclusion that *Rht16* is independent from *Rht18* based on allelism tests. The exact chromosomal location was not determined because of possible misclassification of some lines for height. Thus, further progeny testing is required to confirm the height phenotype of selected F_4 lines. Additional SNP markers are also available on chromosome 5B to fine map this gene once the phenotypic data are confirmed.

6.5 Conclusions

The genetic relationship between *Rht18, Rht14* and *Rht16* has been demonstrated in this study. From the analysis of progeny of intercrosses, *Rht18* is likely to be allelic to *Rht14*, but independent of *Rht16*. Consistent with this finding, *Rht14* was also mapped to chromosome 6AS close to markers that were previously linked to *Rht18*. *Rht16* was mapped to

chromosome 5B through selective genotyping. Coleoptile length assessment showed that *Rht14* and *Rht16* did not reduce coleoptile length. Thus, these dwarfing genes could be of interest in future breeding to reduce height without compromising coleoptile length. This study also provides linked markers for *Rht14* and *Rht16* for marker assisted selection in breeding programs.

Chapter 7 General Discussion

The semi-dwarfing genes *Rht-B1b* and *Rht-D1b* have been exploited extensively in wheat breeding, and have led to substantial yield increases globally (Borlaug 1968; Fischer and Wall 1976). However, these genes are associated with short coleoptiles, and that could affect seedling emergence and stand establishment of wheat cultivars containing the genes under low moisture conditions (Allan et al. 1962; Allan 1980) and where soil temperatures are high (Radford 1987). Therefore, it was desirable to explore alternative height reducing genetic resources to replace *Rht-B1b* and *Rht-D1b*, in another word, to identify new semi-dwarfing genes that may retain high yield without reducing coleoptile length. This thesis compared a promising semi-dwarfing gene *Rht18* with *Rht-D1b/B1b* and other genotypes (Tall and Double dwarf) in the same populations to assess its breeding potential as a semi-dwarfing gene or in combination with *Rht-D1b/B1b*.

7.1 Summary of important traits

Rht18 was compared for agronomic and seedling vigour traits from sowing to harvest in closely related germplasm. Those traits are summarised in Table 7.1 from previous chapters.

Table 7.1 *Rht18* compared with *Rht-D1b/B1b* and Tall for important traits from sowing to harvest. (Values represented by letters indicate the relationship to means, differences ranked as C<B<A, abbreviation: SD, seed dormancy; CL, coleoptile length; SLA, seedling leaf area; SB, seedling biomass; Ant, anthesis date; GNS^{-1}, grain number per spike; GS, grain size; HI, harvest index)

Genotype	SD	CL	SLA	SB	Ant	Height	GNS^{-1}	GS	HI
Rht18	B	B	C	C	B	B	AB	BC	B
Rht-D1b/B1b	B	C	BC	BC	B	B	AB	C	B
Tall	B	B	B	B	B	A	B	B	C
Double dwarf	B	C	C	C	B	C	AB	CD	A

Compared with Tall (Table 7.1), lines with *Rht-D1b/B1b* have a reduced plant height, coleoptile length and grain size but increased grain number and HI to reach higher yields. Compared with *Rht-D1b/B1b*, lines with *Rht18* have increased coleoptile length. *Rht18* delays the growth of spike and distal internodes but it did not change anthesis date. Compared with Tall, *Rht18* slightly reduces plant height but increases grain number and HI to achieve higher grain yield. It may reduce seedling leaf area and biomass but that depends on background germplasm. Grain size in *Rht-D1b/B1b* is always smaller than Tall, but the result at the plant level (Table 2.8 Chapter 2) did not show this difference between *Rht18* and Tall. Double dwarf had shorter height, greater HI but smaller grains as a result of additive effect of *Rht18* and *Rht-D1b/B1b* (Table 7.1). It also further reduced the coleoptile length, seedling leaf area, and seedling biomass affected by either *Rht18* or *Rht-D1b/B1b*.

The clear advantage of *Rht18* over *Rht-D1b/B1b* is its longer coleoptile. Longer coleoptiles allow deeper sowing, which is critically important when the soil moisture is scarce. It is also likely to improve emergence when soil temperatures are warm. Lines with *Rht18* can utilise moisture from deeper soil to germinate and emerge when sown deeper than 5 cm. Kirkegaard and Hunt (2010) showed that in Australia early sowing and other agronomic practices are likely to require wheats with longer coleoptiles and that these will have a significant impact on yield. A yield increase of 14% over a 48-year period and 38% during the millennium drought was predicted by introducing longer coleoptile semi-dwarf wheats. This prediction was from APSIM, a well-validated crop model that is used in Australia (Carberry et al. 2009). Thus, *Rht18* has the potential to play an important role increasing yield in water-limited regions with its long coleoptile particularly when sowing is early. High soil temperatures usually diminish coleoptile length, and lines carrying *Rht-D1b/B1b* sown in such soil have shorter coleoptile length (Botwright et al. 2001a), thus causing poor emergence at shallow sowing. Lines with *Rht18* may still achieve normal emergence when sown in warm

soil. Thus, compared with *Rht-D1b/B1b*, *Rht18* may be more widely adapted in tolerating environmental hardships.

Rht18 was recently reported for its effects on plant height, yield components and coleoptile length in bread wheat (Yang et al. 2015). The paper was consistent with some results from this thesis such as reduced plant height, increased spike stem index and longer coleoptile in lines with *Rht18*. But inconsistencies were also found, some of which were contributed by the approach to the experimental design and selection of germplasm. First, lines with *Rht18* were selected to compare with tall parents or cultivars rather than tall lines derived from each population. The tall parents only represent a line from the same background rather than the averaged values. The tall parents may have advantages in grain yield but have disadvantages in other traits, so the results could be less convincing or misleading from selection bias. Studies reported in this thesis were on contrasting lines developed and enhanced during the project. Care was taken at all stages to compare randomly selected lines in the same genetic background. Thus, experiments in the early stages of the study used replicated random lines of each genotype derived from biparental crosses and later experiments used replicated random lines of backcross derivatives. This resulted in unbiased comparisons between all genotypes. Plant height reductions in different backgrounds from (Yang et al. 2015) were variable ranging from 9% to 25%. In this thesis, *Rht18* was compared with tall lines in different backgrounds (Espada and Young), and the reduction was very consistent, 22% in Espada and 23% in Young. Second, one of the cultivars or parents used in the experiment contains *Rht8*. When comparing *Rht18* with tall parents in this population, the comparison was made between lines with *Rht18* + *Rht8* and *Rh8*. Traits assumed to be affected by *Rht18* were actually the result of interaction between two dwarfing genes. This could be the reason why peduncle length was increased in the short lines of Jinmai47 (*Rht8*) when compared with the tall parent. The coleoptile length was reduced in the short lines in the same background, which is probably the result from *Rht18* in combination with *Rht8*. With

the availability of a *Rht8* marker (Ellis et al. 2005), selection for lines carrying only *Rht18* and lines without any dwarfing gene is possible given the two dwarfing genes are from different chromosomes, and segregate independently.

7.2 Optimum plant height

The optimum plant height for greatest yield (Richards 1992a) for an optimal sowing date is between 70 cm and 100 cm. The semi-dwarfing genes *Rht-D1b/B1b* fall into such a range and they achieve higher grain yields than either short or tall wheats outside this rang (Allan 1986). Among the GA-responsive dwarfing genes, *Rht8* is recognised as a weak height reducing gene because it reduces height by 8% difference. *Rht12* on the other hand is a strong height reduction gene and it reduces height by about 40%, so in most cases it is too short to have the greatest yield. The average height of *Rht18* lines in the multiple experiments in this research was 65 cm in contrast to 87 cm for the tall lines in the Espada background. The height reduction caused by *Rht18* is about 25%, which is equivalent to *Rht-D1b/B1b*.The height of lines in the Espada background when sown at the optimum time was approx. 83 cm, 81 cm, 103 cm, 74 cm for *Rht18, Rht-D1b,* Tall and Double dwarf respectively (Table 2.3 Chapter 2). Height reduction is variable when compared in different backgrounds. Yang et al. (2015) reported the height of *Rht18* in Xifeng20 and Fenchan3 was 72 cm and 83 cm in comparison with their tall counterparts 96 cm and 92 cm. *Rht18* could be a replacement for *Rht-D1b/B1b* since both genotypes achieved the optimum height. The GA-insensitive dwarfing gene *Rht-B1c* is known as a strong height reduction gene which reduces height about 50%, so it is not commercially used. Double dwarf lines containing *Rht-B1b+Rht-D1b* usually ended up with a height similar or even shorter than *Rht-B1c* (Fischer and Quail 1990; Flintham et al. 1997). The Double dwarf lines in this research had an average height of 58 cm, a 32% reduction compared to Tall, which is taller than reported results for *Rht-B1b+Rht-D1b*. Therefore, this

combination should fall into the range of optimum height, as it did in the optimal sowing date, and offers future commercial potential.

7.3 Methods to increase grain yield with *Rht18*

Historically, yield increases were mainly achieved via increased HI or above-ground dry matter (AGDM). From 1972 to 1980, consistent with the introduction of *Rht-B1b/D1b*, yield increases were mainly contributed by a reduction in stem length which resulted in higher HI (Fischer and Wall 1976; Jain and Kulshrestha 1976) and no sacrifice in AGDM. Austin et al. (1980a) suggested that yield increase through genetic gain in improving HI was still possible with the use of shorter genotypes as those genotypes give further reduction in stem and leaves. A theoretical upper limit of HI=0.62 was proposed in the same paper based on assumptions of physiological and mechanical capacity of straw to support extra grains. Like *Rht-B1b/D1b*, *Rht18* in this thesis improves yield through reduced stem weight, and both semi-dwarfing genes increase HI to approx. 0.47 on average in these environments. Double dwarf lines carrying *Rht18 + Rht-B1b/D1b* reduce stem weight further to have higher HI=0.49. A higher HI=0.53 was observed in Riband carrying *Rht-D1b* (Shearman et al. 2005), and HI= 0.61was reported for Consort [RIBAND(SIB)/FRESCO/RIBAND] which was released in 1995 (Spink et al. 2000). Direct comparisons with other HI values are difficult to make as HI varies markedly with sowing date and seasonal conditions. No difference in HI was found here between the HIs of *Rht18* lines and closely related lines with either *Rht-D1b* or *Rht-B1b* and hence there is no reason why *Rht18* lines would not have a higher HI under favourable conditions. It is notable that the combination of *Rht18* and *RhtD1b/B1b* further increases HI without a large decline in plant height. However, Austin et al. (1980a) and Shearman et al. (2005) both pointed out that further improvement in yield through increased HI is limited, as stem and leaves need to provide adequate mechanical support for the spike

and to maximise the light interception. On the other hand, the highest HIs may not always lead to highest grain yields. *Rht-B1c* or Double dwarf lines with *Rht-B1c+Rht-D1b* had the highest HIs in each background, but their grain yields were not the highest (Richards 1992a; Flintham et al. 1997). Similar to optimum height for the highest grain yield, HI within an optimum range is likely to lead to the highest grain yield with the combination of appropriate backgrounds and environments.

Other means to improve yield need to be explored, such as improving AGDM production in high-yielding conditions. Enhancing AGDM has been achieved since 1983 (Slafer and Andrade 1991; Donmez et al. 2001). Studies suggested AGDM progress resulted from greater pre-anthesis growth which is positively associated with radiation use efficiency (RUE) (Calderini et al. 1999; Shearman et al. 2005). RUE is defined as the ratio of dry matter produced per unit of radiant energy used in its production (Monteith 1977). It is positively correlated with leaf photosynthesis (Sinclair and Horie 1989) but negatively with leaf age and respiration (Russell et al. 1989). RUE was not explored in this thesis for *Rht18*, and future work will be likely to explore this area if *Rht18* has any difference to *Rht-D1b* in leaf photosynthesis. Other alternatives to increase AGDM were also reported by increasing fertile tillers number per square meter (Kulshrestha and Jain 1982; Austin et al. 1989), producing larger grains while maintaining the grain number (Calderini et al. 1995), changing the duration of construction phase from terminal spikelet to anthesis (Borràs-Gelonch et al. 2011) or relocating more WSC from stem reserves (Gebbing et al. 1999). In this thesis, no difference was found between *Rht18* and *Rht-D1b* in terms of increasing fertile tiller number or grain size, duration of construction phase or depositing more WSC to grain.

7.4 Future experiments

Rht18 was compared with *Rht-B1b/D1b*, Tall and Double dwarf for physiological traits for the first time. Those comparisons were made between randomly selected lines of each genotype from the same population, rather than by comparing mutant lines with parents or controls. The majority of the populations developed for phenotyping were backcrossed twice to Australian elite cultivars, either Espada (*Rht-D1b*) or Young (*Rht-B1b*), which allowed genotypic comparisons in a uniform genetic background.

The grain yield data presented on a plot basis is closer to the farm environment than expressed per tiller or plant. However, plot data was presented only for one year and it was derived from a biparental population showing variation for yield components. Results from populations backcrossed to Espada in plots are not reported due to bird damage. Those experiments using backcross lines in *Rht-D1b/B1b* need to be repeated in different environments. This will provide informative data on grain yield, HI and above-ground biomass as well as grain size. Plot data and detailed growth studies in the Young background were not available in this thesis, so further experiments in this background should also be conducted to be confident if the results are general or specific to Espada. Additional experiments could be conducted to explore: (i) the value of *Rht18* in the field under deep sowing and in warmer soils where coleoptile length of current semi-dwarfing gene may be penalised. (i) Effect of *Rht18* on early vigour in different genetic backgrounds and different environments. (ii) Whether early floral development is modified in lines with *Rht18* compared with *Rht-D1b/B1b*? (iii) If stem carbohydrates accumulated more in *Rht18*? In the same project, Rebetzke et al (unpublished 2015) compared lines with and without *Rht18* in a different background in field environments in different years. His work showed that *Rht18* reduces lodging but increases grain yield without changing AGDM, and it also increased grain number by producing more grains per tiller as well as more tillers per unit area. Thus,

Rht18 is a promising semi-dwarfing gene in improving yield without compromising coleoptile length in future breeding.

Fine mapping *Rht18*, and the molecular genotyping of segregating populations is the other part of this thesis. Identifying closely linked SNP markers is a key step for mapping *Rht18* and *Rht16* (Chapter 6), which was facilitated by utilising both the 9K and 90K SNP arrays for wheat. The co-segregating SNP marker was developed thanks to the recent construction of a wheat physical map for chromosome 6AS, and this marker was shown to be unique in identifying *Rht18* in hexaploid wheat cultivars. The genotyping platform KASPar^{TM} assay derived from the SNP marker discriminates homozygous lines with or without *Rht18*/*Rht16* in a quick but accurate manner. These technologies will help in future experiments to fine map *Rht16* and characterise candidate genes for *Rht18* and *Rht16*, so that we will understand the basis of their dwarfism.

Root traits such as rooting depth has been studied in conventional semi-dwarf wheats and older tall wheats. However, there is little evidence to suggest that they differ (Hoops 2008). There is some evidence to suggest that *Rht12* may reduce seedling root length (Chen et al. 2013), but no evidence was found in this trait in *Rht18* (Yang et al. 2015). It would be valuable to determine whether GA sensitive wheats, such as those with *Rht18*, has a different root architecture to GA insensitive wheats.

7.5 Breeding potential for *Rht18*

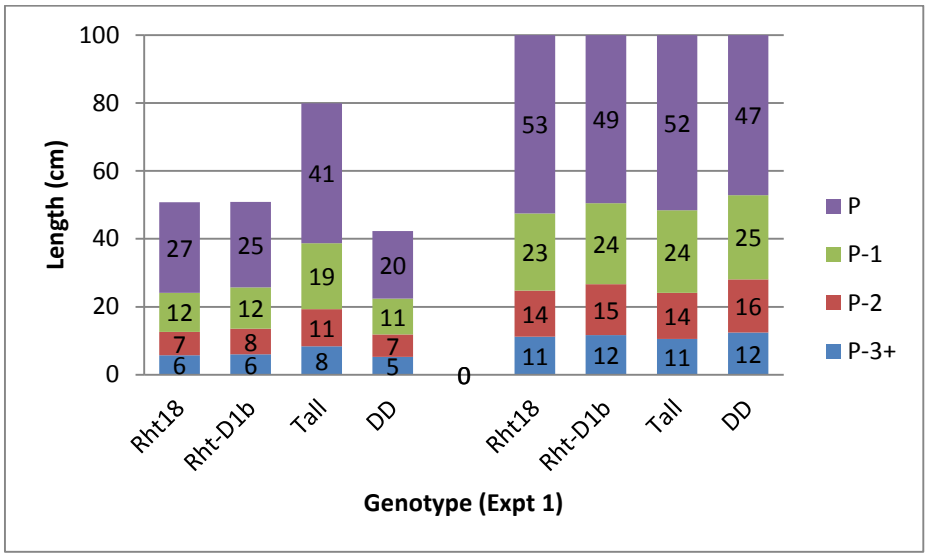
Rht18 is one of the few height reducing genes that have been found outside hexaploid wheat. Initially *Rht18* was discovered in the tetraploid cultivar Icaro, and it was transferred into a hexaploid line HI25M before it was crossed within the hexaploid wheats. HI25M was developed from a cross between hexaploid wheat cultivar Halberd and Icaro. At the early crossing stage, sterility was present in progeny lines and HI25M was selected based on its

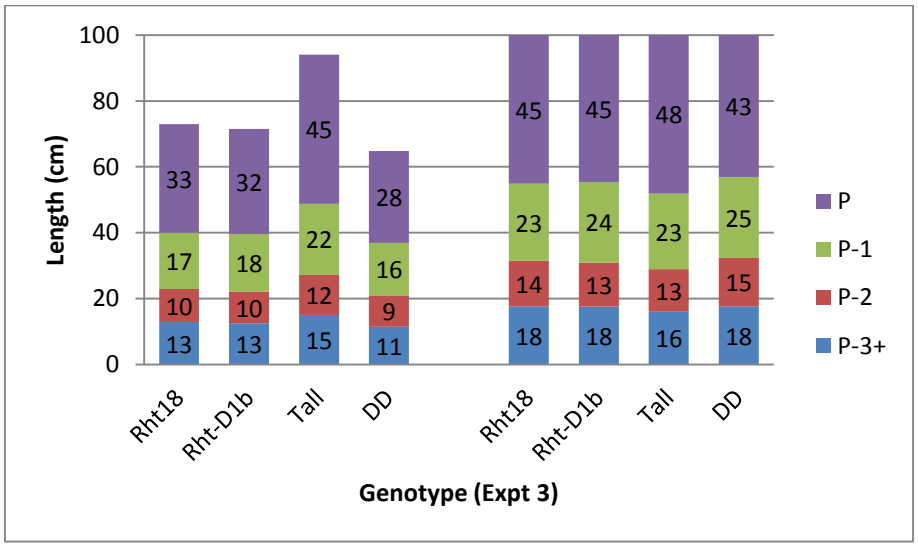
relative higher grain number (pers. comm. Dr. Greg Rebetzke 2014). Thus, before *Rht18* can be used in the following breeding programs, it will be wise to backcross lines with *Rht18* to the parents such as Espada or Young to select for stable and better field performance lines.

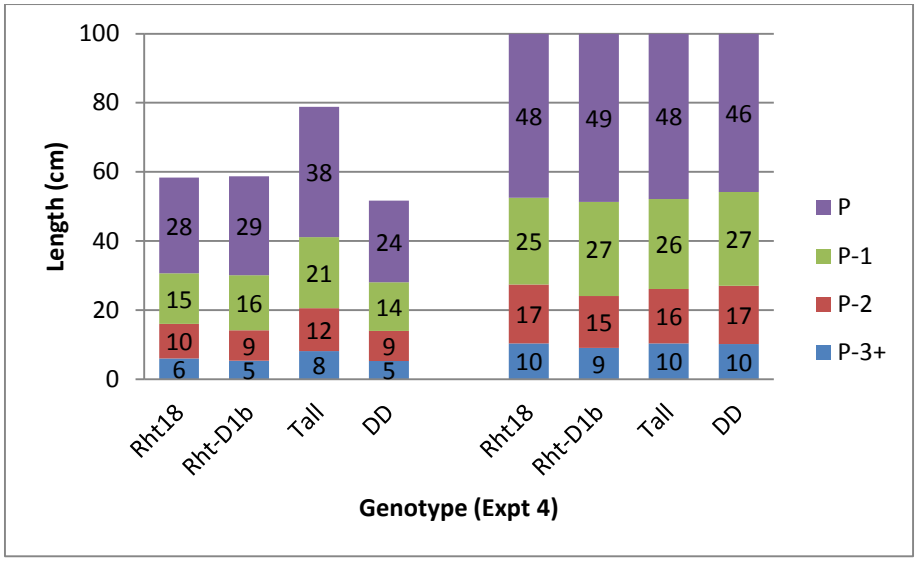
It has been established here that *Rht18* has considerable potential for use in commercial breeding programs. The greatest potential will be in dry environments where sowing may need to be deeper. It will also be important in earlier sowing as soils are usually warmer and potential coleoptile length needs to be longer. Significant advantages, particularly in terms of coleoptile length, were identified. Furthermore, *Rht18* has a phenotype which is easy to select for and robust molecular markers were identified to further enhance its value in breeding. No negative effects were found for *Rht18* to compromise its use in breeding, where it is recommended that it immediately be used in an accelerated backcrossing program where *Rht18* is introduced into the best commercial wheats or breeding lines. Backcross 2 (BC2) lines could be produced which would have, on average, 87.5% of the recurrent parent and therefore are likely to have most of the desirable alleles of the recurrent parent. Markers could be used to select the *Rht18* heterozygote in the BC1 which could then be used for a further backcross. BC2 populations could be grown to select for plants homozygous for *Rht18* with desirable agronomic and disease resistance characteristics, and these could continue to be selfed and selected to conduct yield trials. During this breeding process it would be important to establish whether any negative effects of *Rht18* may be associated with either grain yield or grain quality. In addition, if further reduction in height could be desirable, such as under favourable conditions and where coleoptile length was not important, then both *Rht18* and *Rht-B1b/D1b* could be jointly selected.

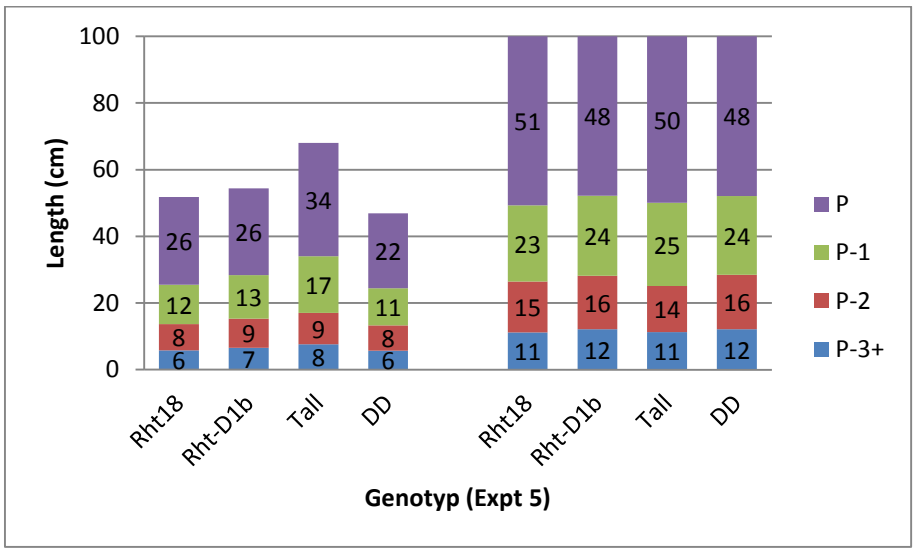
Appendices

Chapter 2









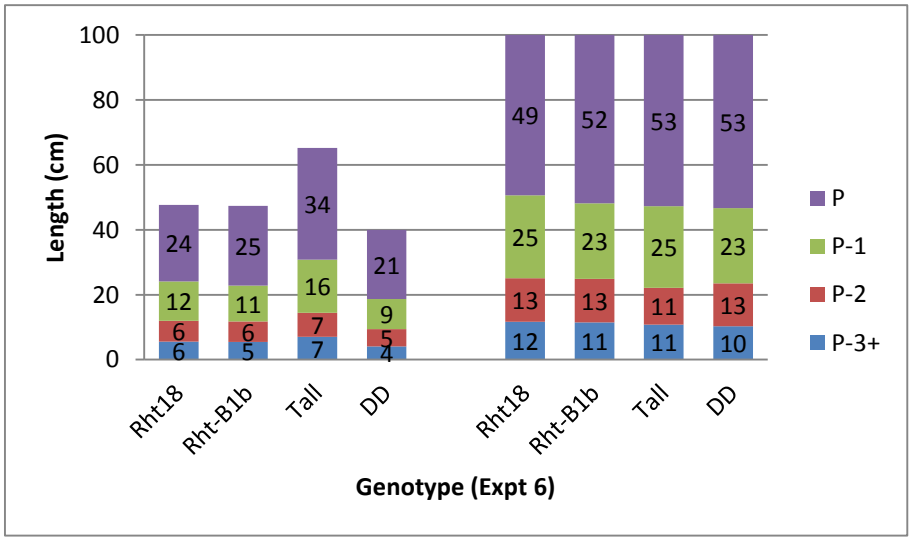


Figure 2.1 Final length and proportion (%) of each internode to the plant height in four genotypes. Expt 1 and 3 sowed in rows at birdcage, Expt 5 and 6 sowed in row at GES, Expt 4: sowed in plots, P: Peduncle, DD: double dwarf, P3+: P3+base internodes.

Table 2.1 Temperature and rainfall records at GES Automatic Weather Station from 2012-2014 (Abbreviation: SM, seasonal mean, averaged over growth period: Sept 2012-Dec 2012, May-Dec in Year 2013 and 2014; LTM, long term mean, averaged over the year; TRF, total rainfall)

Year	2012			2013			2014		
Month	Min. T(°C)	Max. T(°C)	Rain (mm)	Min. T(°C)	Max. T(°C)	Rain (mm)	Min. T(°C)	Max. T(°C)	Rain (mm)
Jan	2.6	33.9	33.2	8	40.7	84.8	7.1	39.2	9.2
Feb	10.1	29.9	189.2	9.7	35.1	86.4	6.9	38.1	96.6
Mar	3.6	26.2	242.6	5.1	30.4	20.4	6.7	27.7	89.8
Apr	0.2	25.7	32.8	0.9	25.9	10.4	1.6	24.7	86.6
May	-2.9	20.9	30.4	-1.7	22.7	12	0	19.8	24
Jun	-3.6	14.7	51.6	-3.2	15	108.2	-0.8	15	74.8
Jul	-4	15	48.4	-3.1	16.4	55.6	-4.1	15.5	27.8
Aug	-3.5	17.6	46	-1.2	18.3	29.4	-4.2	16.2	38.2
Sept	-4.6	21.5	53.2	-0.2	23.5	68.8	-2.1	22.2	43.6
Oct	-0.8	27.4	77.8	-1	30	20	0.1	30.3	57.4
Nov	2.9	34.1	37.2	1.3	31.4	105	2.9	37.7	48.4
Dec	3	34.2	51.2	4.3	37.7	21.2	8	33.1	77
SM	0.1	29.3	49.4	-0.6	24.3	52.5	0	23.7	48.9
LTM	0.3	25.1	74.5	1.6	27.3	51.9	1.8	26.6	56.1
TRF			894			622			673

Table 2.2 Contrast comparisons between each set of genotypes in Expt 2. The data was not significant for six traits.

Genotype	No. of spike	Grain weight (g)	Grain No.	Biomass (g)	Grain yield (g)	Harvest index
Tall vs mean *(Rht18, Rht-D1B)*	ns	ns	ns	ns	ns	*
Rht18 vs *Rht-D1b*	ns	ns	ns	ns	ns	ns
Double dwarf vs *Rht-D1b*	ns	ns	ns	ns	ns	ns

*: $P<0.05$, ns: not significant

Chapter 3

Table 3.1 Analyses of variance for emergence at 3 cm, 9 cm and 12 cm depth in trays

Source of variation	d.f.	m.s.
Genotype	3	174.46*
Treatment	2	30008.56***
Genotype.Treatment	6	280.02***
Residual	36	40.47
Total	47	

*: $P<0.05$, ***: $P<0.001$

Table 3.2 Analyses of variance for emergence at 5 cm and 12 cm depth in field at GES

Source	d.f.	m.s.
Genotype	3	10.192
Treatment	1	13383.6***
Genotype.Treatment	3	10.98
Residual	27	8.988
Total	34	402.641

***: $P<0.001$

Chapter 4

Table 4.1 Summaries of significance for genotypic differences from TS to anthesis in Expt 1 and 3

Population	Trait	Genotype	Thermal time	Genotype × Thermal time
Expt 1	Spike length	*	***	ns
	Spike weight	*	***	ns
	Stem length	***	***	***
	Stem weight	***	***	***
Expt 3	Spike length	ns	***	ns
	Spike weight	*	***	ns
	Stem length	***	***	***
	Stem weight	***	***	***

*: $P<0.05$, ***: $P<0.001$, ns: not significant

Chapter 5

Line ID	Phenotype	*IWA1903*	*IWA1875*	*IWA5238*	*BARC3*	*IWA2457*	*IWA3230*	*WMS4608*	*Rht18*	*csRht18-SNP*	*IWB62878*	*GWM356*	*IWA664*	*IWA6724*	*IWA651*	*IWA1194*
1F8	**short**	A	A	A	A	A	A	A	A	A	B	B	B	B	-	-
1E1	**short**	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B
2G1	**short**	A	A	A	-	-	-	A	A	A	B	B	B	B	B	B
1G5	**short**	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B
1F3	**short**	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B
2A7	**short**	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B
2D4	**short**	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B
1F4	**short**	A	A	A	-	A	A	A	A	A	A	B	B	B	B	B
2E8	**short**	B	B	B	A	A	A	A	A	A	A	B	B	B	B	B
2B8	**short**	B	B	B	B	A	A	A	A	A	A	A	B	B	B	B
1A11	**short**	B	B	B	B	A	A	A	A	A	A	A	A	A	B	B
2C4	**short**	B	B	B	B	A	A	A	A	A	A	A	A	A	B	B
1F7	**short**	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A
1G10	**short**	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A
2G6	**short**	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A
2E3	**short**	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A
1A3	**short**	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A
1F1	**short**	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A
1H10	**short**	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A
1H5	**short**	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A
2A6	**short**	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A
1C6	**short**	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A
1D2	**short**	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A

2B11	**short**	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A
1G7	**tall**	A	A	A	A	B	B	B	B	B	A	A	A	A	A	A
2A9	**tall**	A	A	A	B	B	B	B	B	B	A	A	A	A	A	A
2C9	**tall**	A	A	A	B	B	B	B	B	B	A	A	A	A	A	A
2D10	**tall**	B	B	B	B	B	B	B	B	B	A	A	-	-	-	-
2H4	**tall**	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A
1E10	**tall**	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A
1C10	**tall**	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A
1C3	**tall**	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A
1F9	**tall**	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A
1E6	**tall**	A	A	A	B	B	B	B	B	B	B	A	A	A	A	A
2A8	**tall**	A	A	A	A	B	B	B	B	B	B	B	B	B	B	B
1A10	**tall**	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B
1B11	**tall**	A	A	A	A	A	A	B	B	B	B	B	-	B	B	B
2F1	**tall**	-	-	-	A	A	A	B	B	B	B	B	A	A	A	A

Figure 5.1 Genotyping results of the selected short and tall lines in mapping family.

A=short, B=tall, H=heterozygote, small case means uncertain.

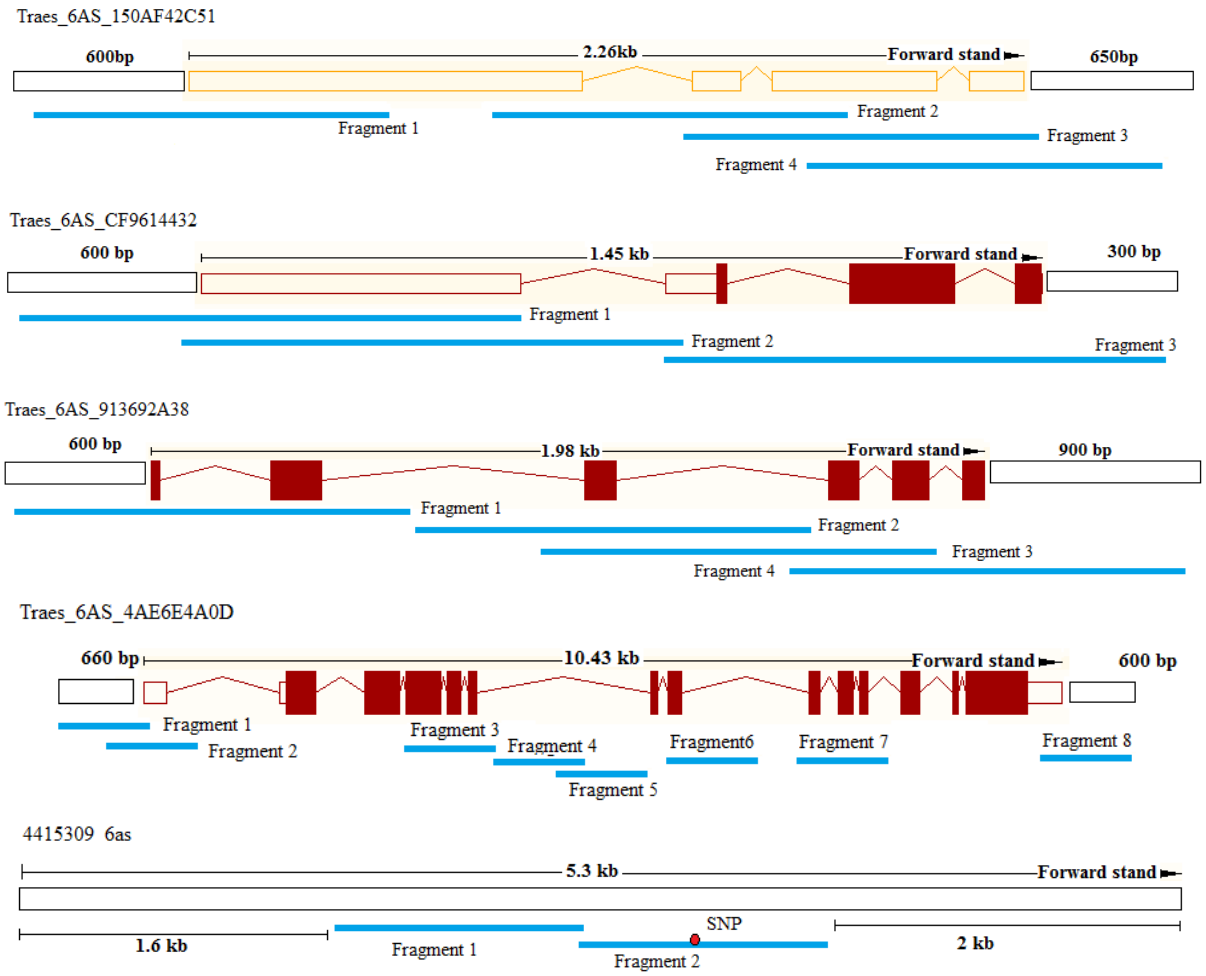


Figure 5.2 Sequenced regions of G1, G2, G5, G8 and contig 4415309_6AS on contig_6AS_1188

▬: Coding exons, White box: flanking sequence contains 5' or 3' UTR (or full sequence of contig 4415309_6AS), Blue bar: Amplified 1 kb region, Red dot: identified SNP

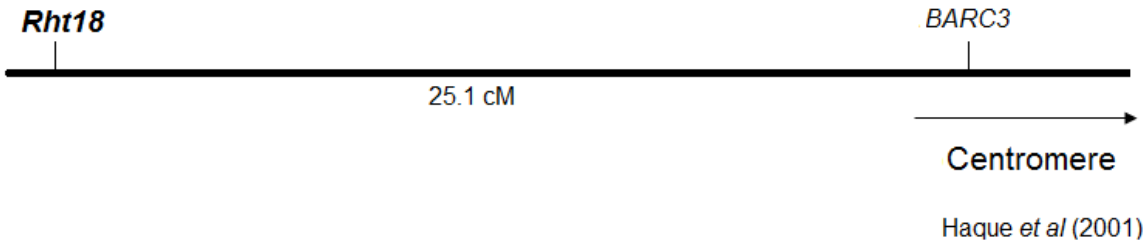


Figure 5.3 Genetic map adopted from (Haque et al. 2011) showing the marker order of *BARC3* and gene *Rht18* on 6AS.

Tables 5.1 and 5.2 have been removed for copyright or proprietary reasons.

Table 5.1 Sequence of Rht18 linked SSR primer on chromosome 6AS

Table 5.2 Sequence of Rht18 linked SNPs or KASPar primers on chromosome 6AS

Table 5.3 Primer and identified SNP information for relevant genes and fragment identified on contig_6AS_1188 between short and tall parents

Gene ID	Transcript length (bp)	Fragment ID	Primers (5'-3')	SNP
G1	1794	1	F: AATGACGTGGGACCTAGATG R: GCTATCCCGGAGCAAGTTTT	no
		2	F: CTGAATCAATTGGCGTCGT R: CCTCTCAATACGTGCCTTTG	no
		3	F: AGCCCTTCTTCACCTATTGC R: AGCTATGGCTCAGCACGTTT	no
		4	F: CTCCATCTCCATCACATCG R: ATGTTCACCTTCGGGCTTCT	no
G2	882	1	F: AGCGGTGTACCGGGATATG R: CTTGGCTGTTGCACTTACGA	no
		2	F: TAGCAGCAGCGTTGGTTTCT R: TCCAAGCTATCAGCAACACG	no
		3	F: GAATTTTCGTAAGTGCAACAGC R: AAAGCAAGCCAATGCAGAGT	no
G5	423	1	F: GTGCCGCATTGGTTGTTATTC R: GCCTGGTTGGGTGACTTTT	no
		2	F: CGCGGAAGTATAGGACGTCAA R: GCAGACAATTGCACTGACAGA	no
		3	F: CACTTCCTCTCCCTGGAAC R: CTTGTTCTCCGGCCAGTTT	no
		4	F: GGCCAGTAGTTGGTTGGAT R: GCGTTTTCTGTCCGTTTACC	no
G6	2752	1	F: AATTTGCTGGTTGGAATGG R: CAAAAACTGAGATCCAGGTGA	no
		2	F: CGTCATTGTTATTGGCATCCT R: TGGTTCTCACTGTGCTGTTGT	S:T T:C*
		3	F: GGGAGACTTGGGTTCTCTGA R: GCACGGAGATTGAGGTTGTA	no
G8	3675	1	F: AGAGGGGTTTGTGGTAACTT R: GCTTCGAAAAGCCTCGATTT	no
		2	F: CTTCCACCCATCTTGTGATAA R: TCCAAATCCTCCAGCAAATC	no
		3	F: ACCTTGCGCTTTCAGGTTTC R: GTAGCTCACGAGCTGGCAAA	no
		4	F: GTACGGGGTATTTCTCCCAGA R: TGCTATCCCATGCTTCAGTG	no
		5	F: ACACTGAAGCATGGGATAGCA R: GAAGCAACATTTGGCTCACA	no
		6	F: GTTGTCTACTCCTGTCCGTCTT R: TCACTTCAATTGGTGCTACCC	no
		7	F: CAAGTGGGTTGGGGAAAAT R: AGTTTTAGCGGGGTTTGGTT	no
		8	F: CTGATATGGTTAGCAGTGCTGT	no

F1	1	R: TGGTTGATGTTGTTGGGATG F: GAACATTTACTGCGTCAGCACT R: CCTCGAACGCACTCAAGAAT	no
	2	F: CAGACGACATCGGTCCTTC R: GAATACTCCCTCCCTTCCTTG	S:G T:C^

F: Forward, R: Reverse, S: Short parent, T: Tall parent

Table 5.4 Phenotypic and genotypic information for lines in the mapping family (Abbreviations: SP, short parent; TP, tall parent).

Generation	Plant ID	Phenotype	Height (cm)	Genotype (*WMS4608*)	*csRht18-SNP*
F_4	1F3	Short	82	A	A
F_4	1G5	Short	91	A	A
F_4	2A7	Short	92	A	A
F_4	2D4	Short	92	A	A
F_4	2E8	Short	92	A	A
F_3	1F4	Short	90	A	A
F_3	2C4	Short	88	A	A
F_4	1A11	Short	100	A	A
F_4	2B11	Short	96	A	A
F_4	2A6	Short	95	A	A
F_4	1H5	Short	95	A	A
F_4	1G10	Short	88	A	A
F_4	2G6	Short	92	A	A
F_4	1H10	Short	96	A	A
F_4	2E3	Short	92	A	A
F_4	1F1	Short	98	A	A
F_4	1D2	Short	112	A	A
F_4	1F7	Short	93	A	A
F_3	1G7	Tall	137	B	B
F_4	2C9	Tall	135	B	B
F_4	1E6	Tall	143	B	B
F_4	1C3	Tall	140	B	B
F_4	1C10	Tall	135	B	B
F_4	1F9	Tall	135	B	B
F_4	2H4	Tall	143	B	B
F_3	1A10	Tall	130	B	B
F_3	1B11	Tall	135	B	B
F_4	2A8	Tall	128	B	B
F_4	1A3	Short	95	A	A
F_4	2B8	Short	112	A	A
F_4	1C6	Short	93	A	A
F_4	1E1	Short	103	A	A
F_4	1F8	Short	93	A	A
F_3	2G1	Short	110	A	A
F_4	2A9	Tall	118	B	B
F_4	2D10	Tall	130	B	B
F_4	1E10	Short	143	B	B
F_3	2F1	Tall	130	B	B
F_4	SP	Short	96	A	A
F_4	TP	Tall	130	B	B

Chapter 6

Table 6.1 Information for *Rht16* linked markers on 90K SNP array

SNP ID	Durum consensus Chr	Durum consensus (cM)	ANOVA *P*-value (Theta)*	Max Theta Diff.^
IWB33231	5B	54.8	2.03E-06	0.28
IWB42727	5B	54.8	1.39E-06	0.39
IWA6837	5B	54.8	2.28E-06	0.42
IWB53020	5B	54.4	9.74E-08	0.43
IWB69519	5B	52.8	6.17E-07	0.62

*: Significant trait linkage when $P < 1E-3$; ^: More likely to detect polymorphism at targeted SNP when value closer to 1

Table 6.2 Sequence of KASP primer converted from SNPs detected from 90K array as link to *Rht16*

ID	Primer name	Sequence
1	IWB33231-FAM/VIC	CTTGAAGTCCGTGAACCTCTCTTT[C/T]
	IWB33231-COM	GAGTGGAGGATATGATCCTATTCAGT
2	IWB42727-FAM/VIC	AGAATATCGGAGCCGAAAAG[A/G]
	IWB42727-COM	TGAGGAGCATTCCTGCTGTA
3	IWA6837-FAM/VIC	GCAAGTTCAACAGCATCACA[A/G]
	IWA6837-COM	CAAGTTGTCAGCACCCAGTT
4	IWB53020-FAM/VIC	TGACAACCACGCAATGTTCC[A/G]
	IWB53020-COM	GGCACAGGAAGAAAGCCTTA
5	IWB69519-FAM/VIC	TCCGGATTTTAGCTTTGTGC[A/G]
	IWB69519-COM	GAAAGCTCGTTGTTCTTCCAG

Base in brackets are the SNPs. FAM/VIC: forward primers, COM: reverse primers.

Table 6.3 Height and coleoptile length in Expt 19 and Expt 20

Line	Height (cm)		Coleoptile (mm)	
	Expt 19	Expt 20	Expt 19	Expt 20
Castelporziano	51.2		102	
Capelli	64.7		135	
Rht14 short	43.7		113	
tall	62.7		116	
Edmore M1		34.8		85.1
Edmore		47.1		123
Rht16 short		36.8		102
tall		49.0		109
l.s.d.	7.3***	2.3***	18.1*	12.9***

*: $P<0.05$, ***: $P<0.001$

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